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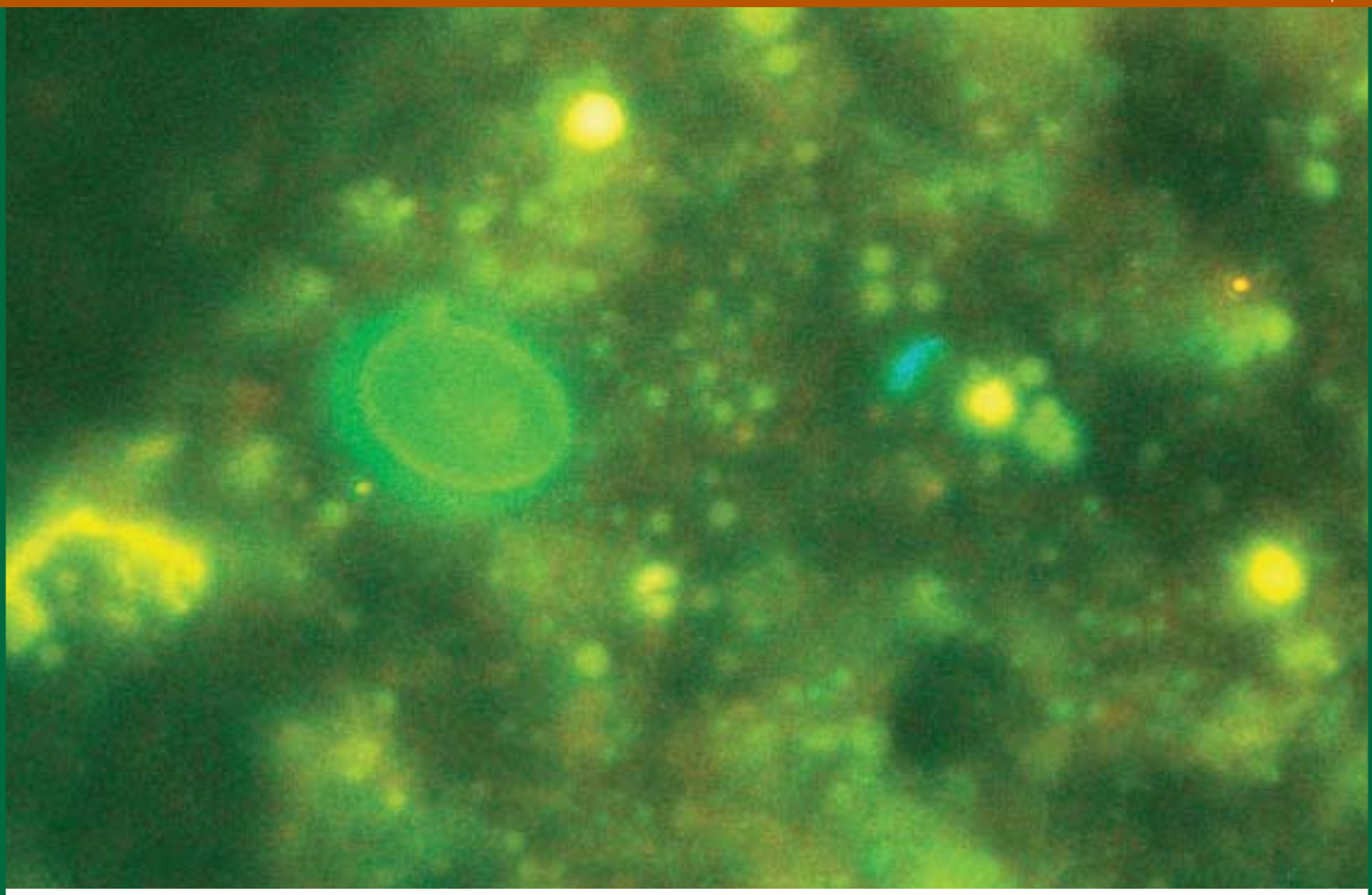

Sewage sludge and sludge products for agricultural use – a study on hygienic quality

(LIVAKE-2001-2002)

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SEWAGE SLUDGE AND SLUDGE PRODUCTS FOR AGRICULTURAL USE – A STUDY ON HYGIENIC QUALITY

(LIVAKE-2001-2002)

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Abstract

The total contents of *Escherichia coli,* enterococci, *Clostridium perfringens* and sulphite reducing clostridia, as well as the presence of *Salmonella*, *Campylobacter*, Shiga-toxigenic *E. coli* (STEC), *Listeria monocytogenes, Mycobacterium*, astroviruses, caliciviruses, *Cryptosporidium* and *Giardia*, were analysed in raw sewage sludge samples taken at 22 sewage water treatment plants, in peat-sludge mixtures, in stabilised (using quicklime, anaerobic mesophilic digestion or in-vessel composting) sludge, and in sludge composts of different ages.

All the microbes studied, except STEC and *Campylobacter,* were common in untreated raw sewage sludge from either rather small communities (a few thousand inhabitants) or large communities (a few hundred thousand inhabitants). After anaerobic digestion, inadequate lime stabilisation and mixing with peat, *Salmonella* and high *C. perfringens* contents were found in all the sludge samples, and caliciviruses, astroviruses, *L. monocytogenes*, *Cryptosporidium* and *Giardia* in most of these samples. After in-vessel composting, *Salmonella* was not found in any of the outfeeds and *E. coli* contents were below 100 cfu/g_{dw} at the drum composting plants. The drum and tunnel composting systems differed in their sanitation ability; in the drum composting outfeeds caliciviruses and cryptosporidia were common, but *L. monocytogenes* was not found, whereas in the tunnel composting outfeeds, caliciviruses and cryptosporidia were not found, but *L. monocytogenes* was common. After further composting in windrows, at 30 weeks all these composts were free of all these pathogens but not of cysts of *Giardia*. Mycobacteria were found in all the composts studied at 10 weeks and in sludge-peat mixtures.

The most resistant pathogens were *Giardia* and *Cryptosporidium*. Their cysts and oocysts were found to survive in windrow composts for 30 weeks even if the sludge had previously been digested or composted in in-vessel plants. *C. perfringens* contents were also high, over log 6 cfu/g_{dw}, in some composts at 30 weeks.

Raw sewage sludge, poorly treated lime-stabilised sludge, digested sludge and sludge-peat mixtures contain a wide variety of potential human and animal pathogens, and thus their use in agriculture is not recommended. It is also recommended that digested sludge and sludge sanitated in in-vessel composting plants should be further composted or cured in open-air windrows or mattresses, with adequate turnings, for at least six months.

Keywords	Sewage sludge, digestion, lime stabilisation, windrow composting, in-vessel composting, sanitation, pathogen, *Escherichia coli,* enterococci, *Clostridium perfringens,* sulphite reducing clostridia, *Salmonella*, *Campylobacter*, Shiga-toxigenic *E. coli* (STEC), *Listeria monocytogenes, Mycobacterium*, astrovirus, calicivirus *Cryptosporidium, Giardia*, PCR
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Tiivistelmä	*Escherichia colin,* enterokokkien, *Clostridium perfringensin* ja sulfiittia pelkistävien klostridien määrät, salmonellojen, kampylobakteerien, Shiga-toxigenisten kolien (STEC), *Listeria monocytogenesin, Mycobacteriumin*, astrovirusten, kalikivirusten sekä *Cryptosporidium*- ja *Giardia*-alkueläinten esiintyminen tutkittiin 22 jätevedenpuhdistamolta otetuista raakalietenäytteistä ja niistä eri tavoin stabiloiduissa lietteissä (kaksi turve-lieteseosta, neljä kalkkistabiloitua, mädätettyä, tunneli - ja rumpukompostoitua lietettä). Lisäksi seurattiin ko. mikrobien esiintymistä jälkikypsytyksen aikana mädätyksen sekä laitoskompostoinnin jälkeen aumatuissa komposteissa että pelkästään aumakompostoiduissa eri-ikäisissä komposteissa. Raakalietteissä - niin suurten kaupunkien kuin pienten paikkakuntien lietteissä - esiintyi yleisesti kaikkia tutkittuja mikrobeja, kampylobakteereita ja STEC-bakteereita lukuun ottamatta. *Salmonellaa* ja suuret määrät *C. perfringens*-bakteereita todettiin kaikissa tutkituissa lietteissä mädätyksen, huonosti tehdyn kalkkistabiloinnin ja turpeeseen seostamisen jälkeen. Näissä lietenäytteissä esiintyi myös yleisesti kalikivirusta, astrovirusta, *L. monocytogenes*-bakteereita, sekä *Cryptosporidium*- ja *Giardia*-alkueläinten kestomuotoja. Laitoskompostoinnin jälkeen ei salmonelloja todettu yhdessäkään ulos purettavassa raa'assa kompostissa. Rumpukompostoinnin jälkeen myös *E. coli* määrät olivat näissä näytteissä alle 100 pmy/g_{ka}. Tunneli- ja rumpukompostoinnit erosivat toisistaan kyvyssään sanitoida puhdistamoliete: kalikivirukset ja kryptosporidien kestomuodot olivat yleisiä rumpukompostoiduissa komposteissa, mutta *L. monocytogenestä* ei niistä löydetty; sitä esiintyi yleisesti tunnelikompostoinnin purkunäytteissä, joista ei sen sijaan kalikiviruksia eikä kryptosporidien kystejä löytynyt. Mistään laitoskompostoidusta kompostista ei 30 viikon jälkikompostoinnin jälkeen löytynyt enää tutkittuja taudinaiheuttajia, lukuun ottamatta giardiojen kystejä. Mykobakteereita oli kaikissa analysoiduissa 10 viikon ikäisissä komposteissa sekä kaikissa turpeen kanssa seostetuissa lietteissä. *Giardiat* ja *Cryptosporidiumit* osoittautuivat tutkimuksessamme kaikkein kestävimmiksi patogeeneiksi. Niiden kestomuotojen havaittiin säilyvän komposteissa jopa 30 viikkoa, vaikka liete ennen aumakompostointia oli joko mädätetty tai laitoskompostoitu. *C. perfringens*-bakteerien määrät olivat korkeita, jopa yli log 6 pmy/g_{ka}, joissakin komposteissa vielä 30 viikon jälkikypsytyksen jälkeenkin. Raaka puhdistamoliete, huonosti kalkkistabiloitu liete, mädätetty liete ja turpeen kanssa seostettu liete sisältävät runsaasti erilaisia potentiaalisia ihmisten ja eläinten taudinaiheuttajia, eikä näitä lietetuotteita tästä syystä suositella käytettäväksi sellaisenaan maataloudessa. Mädätetty liete ja laitoskompostoitu liete tulee jälkikompostoida vähintään kuusi kuukautta kompostiaumoissa tai -patjoissa, ja jälkikypsytyksen aikana on aumojen riittävästä kääntämisestä myös huolehdittava.		
Asiasanat	Puhdistamoliete, mädätys, kalkkistabilointi, aumakompostoinit, laitoskompostointi, sanitointi, taudinaiheuttaja, Escherichia coli, enterokokki, Clostridium perfringens, sulfaattia pelkistävät klostridit, Salmonella, Campylobacter, Shiga-toxigenic E. coli (STEC), Listeria monocytogenes, Mycobacterium, astrovirus, kalikivirus Cryptosporidium, Giardia, PCR		
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PREFACE

In Finland, on average 1,000,000 m^3 of sewage sludge is produced annually. The dry matter content of the sludge is about 16%; thus the amount of the sludge is 160,000 tons as total solids. Mesophilic digestion is a sanitation method for 58,000 tons of the sludge, and of that amount 52,000 tons is also composted after digestion (about 6000 tons is used digested in agriculture). The total amount of composted sludge in year 2000 was 130,000 tons. Composted sludge is mostly, about 80% of the total, documented as being used in green areas. However, a large proportion of this is in fact in storage windrows at composting plants.

The use of sewage sludge and sludge products in agriculture and green areas is controlled by the Ministry of the Environment and by the Ministry of Agriculture and Forestry in Finland. In 1999, a working group called LIVAKE (the working group contributing to the use of sewage sludge and sludge products in Finland), consisting of members from both of the ministries and the inspection centres associated with them was established. One of the main questions to be answered was whether there are any hygiene risks in the use of sludge products in Finland. Thus, the pilot LIVAKE project was started in spring 2000 to monitor the present situation in Finland, and determine whether any further research in this field was needed. These results were published in MMM publication 2/2001. The present study is a continuation of the work of the pilot project.

The project groups express their appreciation to the Ministry of Agriculture and Forestry for financial support for the performing of this work. Further, the researchers thank the workers at all the 22 wastewater treatment plants and associated composting plants for their help during the study.

December 2002

Arja Vuorinen

DEFINITIONS

Raw sludge: a mixture of solid, semi-solid and liquid residues generated during the treatment of domestic sewage in a wastewater treatment plant.

Sludge product: sewage sludge that has been treated to meet the land-application standards (VNp 232/93 and MMMp 46-47/94)

Dewatered sludge: raw or digested sewage sludge in which the water content has been reduced by mechanical means.

Stabilised sludge: sewage sludge, which has been subjected to a stabilisation process, thereby, reducing its tendency to degrade to below a specific level.
Anaerobically stabilised sludge or **anaerobically digested sludge**: sewage sludge produced during anaerobic digestion (temperature, digestion time and the processing system).

Lime stabilised sludge: sewage sludge treated with quick lime (pH, temperature and the treatment time).

Sludge mixed with peat: raw sludge or dewatered sludge mixed mechanically with peat.

Heat dried sludge: dewatered sludge dried to a water content of less than 10% at a temperature of 90°C.

Outfeed compost: dewatered sludge stabilised by in-vessel composting carried out either in batches at tunnel composting plants or in a "plug-flow" system such as drum composting in Finland (temperature, delay time, bulking agent and the composting system). The function of this kind of composting treatment is to stabilise the sludge and to produce a hygienic end product, which further needs composting in open windrows for several months in order to produce a good-quality compost for soil improvement or for use as a constituent for growing media.

Composted sludge or sludge compost: a product which is both stabilised in an in-vessel composting system or in open windrows and further cured for several months in open windrows or mattresses.

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CONTENTS

1 INTRODUCTION

Recycling to agricultural land is an important outlet for sewage sludge and processed sludge products such as compost. In Finland these are used as fertilisers, soil conditioners or main components in growing media. Several treatment processes, or combinations of these, are used for significant reduction of the biodegradability of sludge and its potential to cause harm as well as health and environmental hazards when applied to fields. Thermophilic aerobic and mesophilic anaerobic stabilisation and conditioning with lime are the most common sanitation methods used. After both aerobic and anaerobic stabilisation, sludge is commonly treated further by composting in open windrows. Occasionally, however, sludge may be applied after only a mechanical mixing with peat and lime, without any stabilisation or sanitation.

In this report we discuss sludge treatment methods (lime stabilisation, mixing with peat, stabilisation by anaerobic digestion and composting in open-air windrows, tunnels and drums) exclusively from the point of hygienic view. Stabilisation and maturation are not included. We wish to emphasise that, when sludge products such as composts are sanitised, that is rendered free of pathogens, at the same time they may or may not be stabilised and matured. All these three aspects have to be considered separately, not forgetting that good-quality compost for agricultural use has to fulfil all these three criteria. We also wish to emphasis that in this report we do not discuss phytosanitary aspects of sludge treatment methods.

From an agricultural point of view it must be ascertained that the sludge products do not contain any human, animal or plant pathogens in concentrations that can cause risks to human or animal health, or to food production. Current practices in Finland are based on the requirements of the Act on Fertilizers (LL 232/1993) and the Decision regarding the Use of Sewage Sludge (VNp 282/1994). At the European level, the practices are based on the requirements of the 1986 Directive on the Use of Sewage Sludge in Agriculture (86/278/EEC). Since 1986 new technologies for sludge treatment have been developed, and also more pathogens associated with the food and feed chains have been identified. Even the concerns of the general public relating to acceptable risks have changed. In the EU, a new proposal for a "Sewage Sludge Directive"(3rd Draft of EU Dir. for Sludge Treatment, April 2000), as well as a proposal for a "Composting Directive" (2nd Darft of EU Dir.of Biological Treatment of Biowaste, February 2001) are in preparation, and a Regulation laying down health rules concerning animal by-products not intended for human consumption (1774/2002) was adopted in October 2002.

Pathogens are known to exist in varying numbers in municipal wastewater, depending on their varying prevalence in the community at the given time and the size of the population in the catchment area. They may survive for a prolonged period in sewage water treatment plants and in sludge treatment processes. If the treatment process is incomplete, the effluent from a treatment plant may contaminate the environment, as may also occur when sewage sludge or inadequately processed sludge products are used as fertilisers. Direct or indirect transmission of zoonotic agents – pathogens that can be transmitted between animals and humans – to farm animals is generally regarded as one of the most relevant risks in connection with untreated or insufficiently processed products (Böhm 2002). However, direct and indirect transmission to humans via sludge products also has to be regarded as a potential risk and is of special importance when the chain from the field to the table is concerned. Among the zoonotic agents, salmonella, campylobacter, listeria, Shigatoxigenic *Escherichia coli* (STEC), viruses, eggs of helminths and enteric protozoa are of particular interest when sewage sludge is used in agriculture, private gardens and green areas. The infectious dose of some of these pathogens may be as low as one particle (virus) to 50 organisms (parasite) and 10-100 bacteria (STEC). When sludge is applied to

land for agricultural use or when sludge compost is used as a soil improver, these pathogens may survive from days (bacteria) to months (bacteria, viruses) to years (helminthes eggs), depending on the environmental conditions (Straub et al. 1993). The number of animal mycobacteria in sewage is not known. Mycobacteria, as well as the zoonotic human pathogens and viruses mentioned above do not belong to the intestinal flora, but they may be found in faeces in clinical cases.

Indicator microbes are commonly used for routine monitoring of the performance of a sewage water treatment plant and the quality of the processed sludge. Indicator microbes should commonly be found in raw sludge in high numbers, be easily detectable and their resistance to sludge treatment processes should be similar to or better than that of pathogens. *E. coli*, enterococci and *Salmonella* spp. are the most common bacterial groups suggested for indicator organisms (Strauch 1987, Haug 1993, 3rd Draft of EU Dir. for Sludge Treatment, April 2000), but *Clostridium perfringens* (Carrington 2001; 2nd Draft of EU Dir. for Biological Treatment of Biowaste, February 2001), sulphite-reducing clostridia (Böhm 2002) and *Salmonella* together with *Enterobacteriaceae* (Reg.1774/EU 2002) have also been proposed. Densities of these microorganisms in primary sludge are reported to be 10^2-10^3 colony forming units (cfu)/g for *Salmonella*, 10^6-10^7 cfu/g for faecal streptococci, 10^8-10^9 cfu/g for total coliforms (Straub et al. 1993) and 10^6 cfu/g for *E. coli* (Davis et al. 1999, cited in Carrington 2001). Carrington (2001) suggested that numbers of *E. coli* should not exceed 1000 cfu/g_{dw} (dry weight) and it is recommended that spores of *C. perfringens* should not exceed 3000 cfu/g_{dw} in the final sludge product (Carrington 2001) or should be absent in 1 g of compost/digestate (2nd Draft of EU Dir. for Biological Treatment of Biowaste, Feb. 2001).

Bacteria are known to have a capacity to acquire resistance against antibiotics and other drugs. Resistance has a genetic basis; bacteria can exchange this genetic information by either mutation or gene acquisition, or by both. Transmission of resistance factors is possible both between bacteria belonging to the same family and between different bacterial groups. Two important locations where resistant strains are built and selected are the digestive system of humans and animals, and the environment (water, soil, animal litter, sewage, hospital premises, etc.; Acar & Röstel 2001). Bacteria are defined as multiresistant if they are simultaneously resistant to three or more classes of antibiotics. Resistance of bacteria has increased with the increased use of antimicrobial agents in human and veterinary medicine and for growth promotion or in prophylactics in food producing animals, and it may seriously interfere with the successful treatment of infectious diseases.

Salmonellae, including about 2500 serotypes, are the most relevant pathogenic bacteria, since they can infect a wide variety of animals, including humans. Salmonellae are globally distributed and commonly cause foodborne outbreaks in both industrialised and developing countries. All mammals, birds and reptiles can be asymptomatic carriers of salmonella and can shed them to faeces intermittently at varying concentrations (Himathongkham et al. 1999).

The prevalence of salmonella in both domestic and wild animals in Finland is low. The goal of the National Salmonella Control Program in Finland is to keep the level of salmonella below 1% in cattle, pigs and poultry, as well as in beef, pork and eggs. This low level is also reflected by a low incidence (about 10/100,000/year) of indigenously acquired human salmonelloses in Finland (statistics of the Laboratory of Enteric Pathogens, National Public Health Institute [KTL]). Thus at least 80% of all about 3000 cases of salmonellosis annually have been associated with recent trips abroad or been caused by serovars not endemic in Finnish animals. Salmonella infections also cause reactive arthritis as a post-infectious complication in about 10% of infected humans (Mattila et al. 1994, 1998).

Campylobacter spp. is an enteric pathogen causing gastrointestinal illnesses in humans of all age groups. Since the 1990s, the number of reported campylobacter infections has annually increased in most of the western European countries (Rautelin & Hänninen 2000). In Finland, approximately 3900 cases were reported in 2001

(National Infectious Disease Registry [NIDR], National Public Health Institute), exceeding the number of reported salmonella cases. Campylobacters are zoonotic organisms, and many animal species can serve as reservoirs for human infections. Owing to their microaerophilic nature and a high minimum growth temperature (approximately 30°C) they do not multiply in the environment as do many other enteric bacteria. About 50 campylobacter infections per 100,000 people are found in Finland annually (NIDR). These bacteria are common in both influent and effluent sewage water as well as in sewage sludge, but they have not been found to survive in sludge treatment processes (Jones 2001).

Shiga toxin-producing *Escherichia coli* (STEC) is a serologically diverse group of *E. coli* bacteria, able to produce either one or both Shiga toxins (Stx1, Stx2) encoded by the corresponding *stx* genes. Bloody diarrhoea is typical of a STEC infection. The clinical picture of the infected subjects may, however, vary from an asymptomatic state to life-threatening complications, such as haemolytic uremia syndrome (HUS) or thrombotic thrombocytopaenic purpura (TTP). Of the STEC serotypes, O157:H7 has occurred most commonly worldwide. However, a total of approximately 250 non-O157 STEC serotypes have been reported, and more than 100 of them have been associated with human illness. Since 1995, about 190 STEC infections have been identified in Finland (Eklund et al. 2001; Saari et al. 2001; unpublished data). At present, the incidence of all STEC infections in Finland is below 0.3/100,000/year (NIDR). The main reservoir of STEC has appeared to be cattle, which may shed the bacteria through milk and faeces without any clinical signs. In Finland, the prevalence of O157:H7 strains in cattle has been 1.3% (Lahti et al. 2001), and identical strains have been isolated both from the cattle on farms and from subjects who had visited these farms (Lahti et al. 2002a). The specific sources of the non-O157 infections in Finland have remained unclear. STEC O157 strains have an ability to survive in soil, water and manure; treatment of cattle slurry by anaerobic digestion and aeration of manure piles may reduce its viability (Maule 2000). Lettuce fertilised with manure or irrigated with water with *E. coli* O157:H7 contamination can take up the bacteria through its root system and internalise them inside its leaves (Solomon et al. 2002).

Listeria monocytogenes is omnipresent, and its natural niche is thought to be soil and vegetation (Gram 2001). It is resistant to diverse environmental conditions and has a good ability to survive for long periods in the environment (soil, plants and water), and it can enter the food chain through the environment. It is able to infect both humans and animals. Infected persons usually belong to a certain risk group with an immunosuppressive condition or medication, and the infection can be life threatening. For healthy adults the level reported to have caused infection has varied from 10^5 to 10^7 cfu/g; for risk groups the level has been lower (<10^4 cfu/g or even <10 cfu/g) (Maijala et al. 2001). Two to 10% of healthy people are reported as being asymptomatic faecal carriers of *L. monocytogenes*; in Finland there are annually 0.6-1 human infections per 100,000 people (NIDR). In animals the clinical picture can vary, and the susceptibility to listeriosis differs from species to species. Silage of minor quality is an important source of infection in farm animals (Pell 1997). Mac Gowan et al. (1994) found *L. monocytogenes* in 60% of sewage specimens, including untreated liquid sewage, the levels being less than 50 cfu/ml.

Environmental mycobacteria can grow in water and soil. More than 90 nontuberculous mycobacteria (NTM) species have been described. They have been recovered from a wide variety of environmental sources, including water (hot and cold water taps, ice machines, heated nebulizers and showerheads), soil, dust, aerosols and animals. Most of them are saprophytic, although some are potential pathogens and may be involved in pulmonary or cutaneous diseases or in lymphadenitis. Infection due to NTM is increasing in both industrialised and developing countries.(Falkinham III 1996, Falkinham III et al. 2001, Iivanainen et al. 1993, Iivanainen 1995, Iivanainen et al. 1997, Iivanainen et al. 1999, Torkko et. al. 2000, Vuorio et al. 1999, Wolinsky et al. 1992). The human NTM

infection rate is approximately 1/10,000 (NIDR 2001) per year in Finland. The majority of animal mycobacteriosis cases are found in pigs, prevalence approximately 0.34% in Finland. *M. avium-M. intracellular* complexes (MAC) are predominant species in both human and animal cases. Medical therapy of mycobacteriosis is difficult and not always successful. NTM are usually resistant to isoniazid and rifampicin, the two most important agents for the treatment of mycobacteriosis. Furthermore, the conditions responsible for the survival of mycobacteria in water and soil are not well investigated.

Although there have been reports on the presence of environmental mycobacteria in water and soil distribution systems, no studies have been published so far concerning the detection of mycobacteria in sewage sludge and sludge products by using molecular methods.

The emphasis in research on the survival of **viruses** during sewage treatment has largely been on plant (e.g. Lopez-Real & Foster 1985, Bollen 1985) or animal (Lund 1979) pathogens, rarely on human (see Straub et al. 1993) pathogens. Both bacteriophages and plant viruses have been studied as indicators of faecal contamination (Christensen et al. 2001, Johansson et al. 1997). Little attention has been given to the possible presence of human pathogenic viruses in sludge and their survival in sludge treatment processes, although some authors have suggested their monitoring (Bardos et al. 1992, Wyn-Jones & Sellwood 2001).

Norwalk-like viruses (NLVs, Noroviruses) belong to the family Caliciviridae and cause diarrhoea in humans of all ages. As is the more pathogenic hepatitis A virus, NLVs are highly resistant in the environment and may cause water- and food-borne disease. Thus, they can be regarded as relatively good indicators of the sanitation of viral pathogens. Human astroviruses cause gastro-enteritis mainly in children. Despite the limited population secreting astroviruses, their concentration in sewage may be considerable as a consequence of outbreaks (v. Bonsdorff et al. 2002b).

Unlike bacteria, human viruses are incapable of multiplying outside human cells. Thus, their presence in sewage water is solely dependent of the survival of their initial seedlings into sewage. A large proportion of the viral load is in most instances and most waste treatment plants evidently released in the effluent sewage into the receiving watercourses. In sewage water they may reach considerable concentrations during endemic seasons (see v. Bonsdorff et al. 2002a). At present, the role of enteric viruses as causative agents of outbreaks related to contaminated water (e.g. fresh produce, bivalve molluscs) is being studied increasingly (see Koopmans et al. 2002).

Giardia and ***Cryptosporidium*** are protozoan parasites causing diarrhoea in humans all over the world. In industrialised countries, Cryptosporidia have been found in 12% of the faecal samples studied (Guerrant 1997). The incidence of *Giardia* infections has been shown to vary from 2% to 5% in industrialised countries (Ortega & Adam 1997). In Finland, approximately 300 cases of giardiasis and 10 cases of cryptosporidiosis have been reported annually (NIDR). There is no curative therapy for cryptosporidiosis (Clark 1999). Symptomatic individuals may excrete 10^5-10^7 *Cryptosporidium* oocysts per gram of faeces (Fayer 1997). During wastewater treatment, oocysts and cysts are precipitated in sewage sludge, where counts of 10^5-10^6 cysts per kg have been detected (Straub et al.1993).

During the past few years, several review articles evaluating the hygienic aspects of organic waste used in agriculture (Albihn 1999), the quality of composts including the reduction of pathogens (Johansson et al. 1997), and the sludge treatments for pathogen reduction (Carrington 2001) have been published in order to determine criteria for controlling the hygienic quality of the product. Christensen et al. (1999, 2001) investigated with indicator bacteria and plant pathogens the sanitary quality of composts at four full-scale composting plants in order to develop a Nordic system for evaluating the sanitary quality of composts. However, there are still no studies evaluating at the same time the survival of both indicator bacteria and bacterial, viral and protozoal human pathogens in full-scale sludge treatment plants. Methods for determining pathogens in sludge and sludge

products have been inadequate, and their sensitivity to pathogens has not always been adequate. Application of molecular methods for detection may improve the sensitivity and specificity of the methods. Detection of certain human viruses, such as caliciviruses, has been significantly improved by the use of RT- PCR.

2 AIMS

The aims of this study were

1. to investigate the survival of human and animal pathogens in various sewage sludge treatment processes used in Finland, the products of which are commonly used in agriculture, private gardens or green areas
2. to determine which indicator microorganisms would be suitable for microbiological safety monitoring of sludge products
3. to determine whether the analysis methods chosen were appropriate/suitable for the enumeration or detection of the microbes chosen as indicators
4. to produce more background data for legislation at the national as well as at the European level.

3 MATERIALS AND METHODS

3.1 Experimental design

Twenty-two full-scale wastewater treatment plants were selected for the research. The selection was based on the sludge treatment method used at each given plant. The aim was to find four representatives for all the six most common Finnish sewage sludge processing methods, but only two plants (plants 9 and 10) were found where the mixing of sludge with peat was the sole sludge treatment method. Plants 11 and 12 are thus missing. Schematic descriptions of the plants are presented in Table 1 (Appendix 1) and Figure 1, and their functions briefly in Appendix 2.

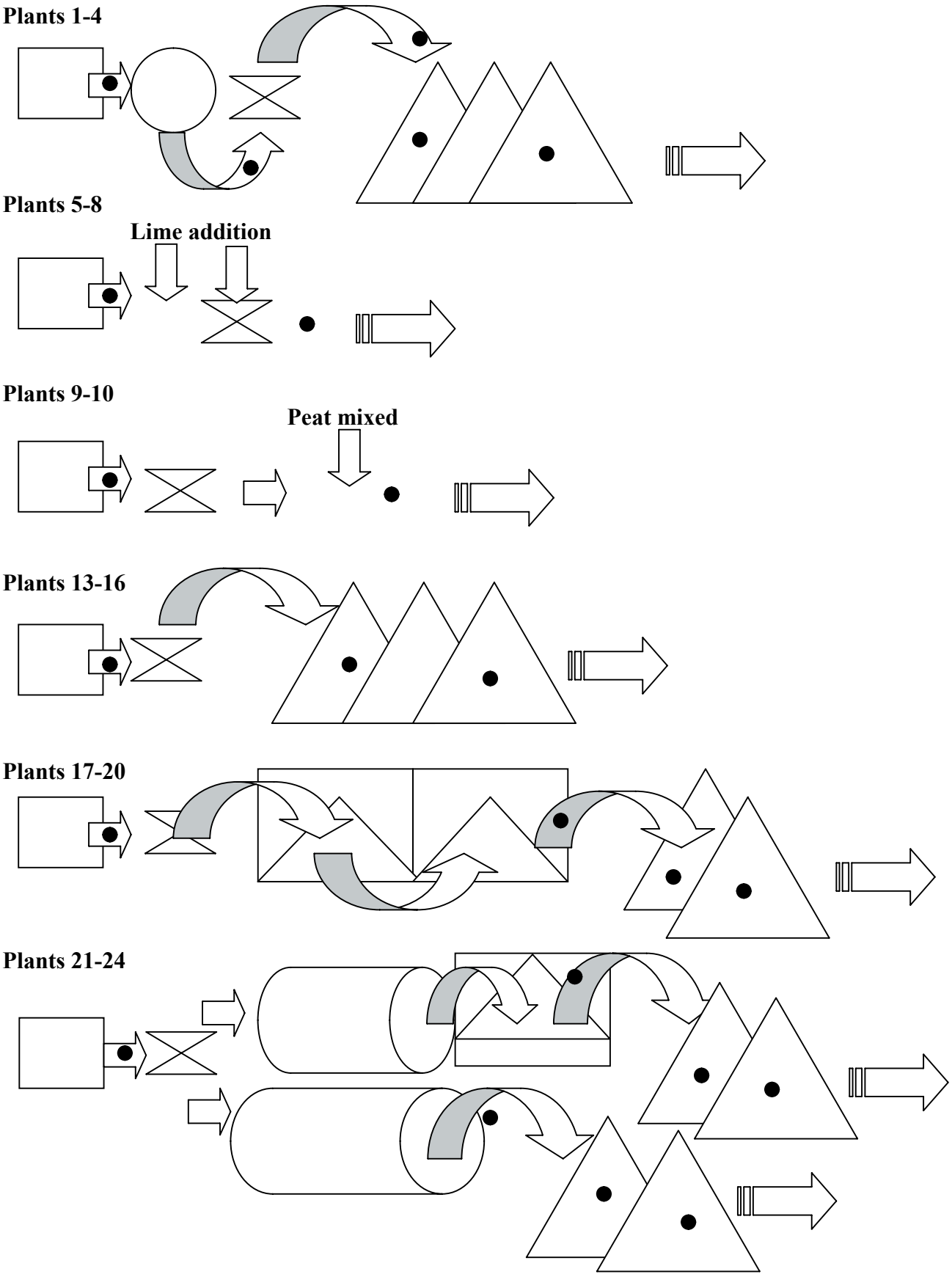
3.2 Sampling and sample processing

Samples from raw sludge (mostly a complex mixture of primary, secondary and tertiary sludge) were collected during three successive days (7 samples of one litre per day; one sample per laboratory, except at plants 5 and 16, where only two successive samples were taken). In the participating laboratories these three samples were mixed 1:1:1 in order to obtain one composite sample from each plant. However, all three samples were investigated separately for STEC O157, *L. monocytogenes,* and for *Giardia* and *Cryptosporidium*, and only one of the three successive samples was analysed for the presence of viruses. The sludge sample collected on the 2nd day was recorded as one-day-old sludge (see Table 1). After anaerobic digestion and lime stabilisation the samples (7 samples of one litre) were taken in such a way that they represented the same proportion of the sewage as did the raw sludge samples (the "age" of the sludge of each sample is seen in Table 1). Samples from the peat-sludge mixtures and the composted sludge were taken as composite samples (30 litres) from heaps or windrows, from 20 spots at a depth of 50 cm or from 20 random spots from the fresh mechanically made surface in the windrow or mattress). After mixing the composite samples were divided into seven equal portions of 3-4 litres, one subsample for each laboratory. They were refrigerated during transportation and in the laboratories prior to analysis.

Dry and organic matter content as a loss of ignition (LOI; EN 13039:1999); pH (EN 13037: 1999; liquid samples measured without extraction) and conductivity (EN 13038; liquid samples not measured) were measured for all samples.

That temperature was measured manually at a depth of 1.5 m from a level of 1.5 m at the time of compost sampling. In most of the in-vessel composting plants, temperatures of the composting mass or the outlet air were measured during the active process (see Appendix 2).

Figure 1 The process schemes of the wastewater treatment plants (1-24) and the sludge treatment systems; sampling marked by black spots (•) in the scheme.



3.3 Microbiological methods

3.3.1 Indicator bacteria

For the quantitative analysis of the indicator bacteria, samples of 11 g were suspended in 99ml of peptone water, were homogenised in rotary shaker at approximately 150 rpm for 5 min and were serially diluted for plating out.

Clostridium perfringens was analysed according to a modified ISO 7939 (1997) method. Samples were serially diluted in a solution of peptone (0.1%) and saline (0.85%) and were inoculated into TSC (Tryptose-Sulphite-Cycloserine agar, Oxoid, Hampshire, UK) agar using the pour-plate technique. The plates were incubated under anaerobic conditions at 37°C for 20±2 h. Typical colonies were isolated and inoculated onto sheep (7%) blood agar plates (Oxoid) and were incubated under anaerobic conditions at 37°C for 18-24 h. Haemolytic colonies were confirmed by Gram staining and API 20 A (Analytical Identification Profile, bioMeriéux, France).

Sulphite-reducing clostridia were analysed by a modified NMKL 56 (1994) method. Samples were serially diluted and were inoculated into TSC agar by using the pour-plate technique and were incubated under anaerobic conditions at 37°C for 18-24 h. Confirmation included Gram staining and a catalase test.

Analysis of **enterococci** was performed using a modified method based on NMKL 68 (1992). Samples were plated out on Slanetz and Bartley medium (Oxoid) and were incubated at 44°C for 48 h and red colonies were counted. Confirmation tests included Gram staining, a catalase test and an API 20 Strep test. For the antibiotic resistance test, five colonies per sample, containing enterococci were selected. Enterococci in 30-day old compost samples were excluded. The antibiotic resistance test on the isolated enterococci strains was a modification of the method described in the publication of NCCLS (National Committee for Clinical Laboratory Standards. 1998. Performance standards for antimicrobial susceptibility testing; Eighth Informational Supplement. Document M100-S8, vol:18; Number 1. NCCLS, Wayne, PA.).

Escherichia coli was analysed by a modified method of NMKL 147 (1993). Samples were inoculated onto Petrifilms and were incubated at 37°C for 46-50 h. Blue colonies were confirmed using an API 20 E test.

Salmonella was analysed using a modified ISO 6579 (1993) method. In brief, 25 g of the sample material was pre-enriched in 225 ml of buffered peptone water (Merck, Darmstadt, Germany) at 37°C for 16-24 h. Two selective enrichment broths were used: 0.1 ml of the pre-enrichment culture was transferred to Rappaport-Vassiliadis soya peptone broth (IDG, Bury, Lancashire, UK) and was incubated at 41.5°C for 18-24 h, another dose of 1 ml of the pre-enrichment culture was transferred to selenite-cystine broth (Merck) and was incubated at 37°C for 18-24 h. Each of the selective enrichment cultures was plated out on three selective solid media, xylose-lysine-desoxycholate agar (XLD, two parallel plates, Merck), bromthymolblue agar (in-house made) and Rambach agar (Merck). The plates were incubated at 37°C for 18-24 h. Confirmation of the colonies was done in triple sugar-iron agar (TSI; in-house made) tubes, using anti-O serum (omnivalent, Dade Behring, Marburg, Germany), and in API 20 E. Serotyping was carried out by slide agglutination. The O-antigens were assayed from TSI agar, and for H-antigens, growth taken from either TSI agar, semi-solid nutrient agar or motility agar was used (WHO Collaborating Centre for Reference and Research on *Salmonella*, Popoff M.Y. & Le Minor L. 1997. Antigenic Formulas of the Salmonella Serovars). The antimicrobial susceptibility of the isolated *Salmonella* strains was determined according to NCCLS.

3.3.2 Other pathogenic bacteria

Listeria monocytogenes was analysed by enrichment according to ISO 11290-1 (1996) but modified by using *L. monocytogenes* blood agar medium (LMBA) (Johansson 1998) improved by an addition of magnesium sulphate (anhydrous) 2.5 g/l and nalidixic acid (40 mg/l) (Johansson 2001, personal communication) as selective plating

medium instead of Oxford agar. In brief, samples of 25 g were enriched in 225 ml of half-strength primary enrichment broth, half Fraser broth (Oxoid) at 30°C for 24 h. Volumes of 100 µl of the primary enrichment cultures were transferred to 10 ml of the full-strength secondary enrichment broth, Fraser broth, and were incubated at 37°C for 48 h. Both of the enrichment cultures were plated out on PALCAM medium (Merck) and improved LMBA (in-house made). The plates were incubated at 37°C for 24-48 h. Confirmation was based on detection of β-haemolysis (trypticase soy agar, BBL, supplemented with 5% defibrinated bovine blood), a catalase test (3% hydrogen peroxide solution), Gram stain and the API *Listeria* test. The detection limit of the method was for raw sludge and for outfeed 30 cfu/25g, and for cured composts 3 cfu/25 g (for validation data, see Appendix 3).

Mycobacterium Compost samples of 4 g were incubated in 40 ml of TSB broth (Tryptic Soya Broth BBL, Becton Dickinson & Company, Cockeyville, USA) for 25 min. Finally, in order to remove the maximum number of microorganisms from their organo-mineral substrates the sample suspensions were centrifuged (500 g at 4°C, 5 min) (Iivanainen et al. 1997). Isolation of mycobacteria was performed as recommended by Iivanainen (1995). In brief, the centrifuged supernatant was pre-incubated at 37°C for 5 h, followed by decontamination with NaOH (2-mol/ l) for 20 minutes. Centrifugation was repeated at 1000 g for 20 minutes. Thereafter the supernatant was decontaminated with oxalic acid (5% w/v) for 20 minutes, and 100 µl was inoculated onto Middlebrook 7H10 and mycobacterium 1 and 2 tubes (Orion Diagnostica, Espoo Finland). The detection limit of the method was 10^4 cfu/g of dry soil, but even 10^8 cfu/ml was below the detection limit in raw sludge samples.

The identification of mycobacterial isolates was based on the method of Ziel-Neelsen and 16S rDNA-gene sequencing. Colonies were stained by the Ziel-Neelsen Carbol Fuchsin Stain. Representatives of each acid-fast colony type were subcultured on Middlebrook 7H10 agar plates (Difco Laboratories, Detroit, USA) were incubated at 28°C and were stored in TSB-glycerol broth (BBL, Bencton Dickinson & Company) at –70°C. The DNA extracts were made from pure cultures of isolates grown on Middlebrook media. A 10-µl loopful of culture was mixed in 1 ml of sterilised H_2O and was heat killed at 95°C for 10 min. Five freeze (liquid N_2) - thaw (80°C) cycles were performed, after which the suspensions were centrifuged (5 min, 11,000 x g) and the pellets were re-suspended in 300 µl of TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Glass beads (diam 0.1 mm) were added and the cells were mechanically disrupted in Vibrogen Cell Mill (Edmund Bühler, Germany) for 2 min. DNA extract was separated from the beads by centrifugation (5 min, 9000 g) and was frozen for sequencing. A 500-bp fragment from the 5' end of the 16S rDNA gene was amplified and sequenced using a MicroSeq 500 16S rDNA kit (PE Applied Biosystems, USA) and an ABI PRISM 310 genetic analyser (PE Applied Biosystems) as described by Patel et al. (2000). The obtained sequences were edited using Sequencher™ 3.0 software. To identify the strains, the sequences were compared with RIDOM - Ribosomal Differentiation of Medical Microorganisms (Harmsen et al. 1999), EMBL (Stoesser et al. 2001) and NCBI-GenBank (Benson et al. 1997) databank sequences. The detection limit of the method was 10^4 cfu/g of dry soil, but even 10^8 cfu/ml was not detectable in raw sludge.

PCR for STEC At first, a loopful (10 µl) of each sewage sample was cultured directly onto Sorbitol MacConkey (SMAC) agar plates, and 10 g was suspended in 90 ml of Trypticase Soya Broth (TSB; Difco, Sparks, MD) with 20 µg/ml novobiocin (Sigma, St. Louis, MO) (mTSB; Heuvelink et al. 1998, Heuvelink et al. 2000). The cultivations were carried out in duplicate. The PCR runs for the detection of the stx_1 and stx_2 genes were executed as previously described (Keskimäki et al. 1998; Eklund et al. 2001[KTL-EHEC 04a, 04b]). However, of the mTSB cultures, 500 µl was boiled for 15 min, and was centrifuged for 15 seconds, and 1.5 µl was used as a template. The detection limit of the method was 10^4 cfu/g of sewage with scanty growth and 10^5 cfu/g of sewage with abundant growth (for validation data, see Appendix 3).

Immunomagnetic separation (IMS) specifically used for STEC O157 . The method used was a modification of ISO 16654:2001. Samples of 25 g were added to 225 ml of pre-warmed (37°C) modified Trypticase Soya Broth (LAB M O157 broth MTSB) with Na-novobiocin 20 µg/ml (Sigma) and were incubated at 41.5°C. IMS from MTSB-culture broth was performed at both 6 h and 24 h by using Dynabeads anti-*E. coli* O157 (Dynal A.S., Oslo, Norway) according to the manufacturer's instructions. Fifty microlitres of the IMS-complex was spread onto sorbitol MacConkey agar plates without (SMAC, LabM) or with cefixime-tellurite supplement (CT-SMAC, LabM). The plates were incubated overnight at 37°C. Sorbitol-negative colonies were tested for the production of β-glucuronidase, raffinose, dulcitol and indole by standard techniques. The colonies were further identified as *E. coli* by using API 20E. Serotyping of the O157 was performed using slide agglutination tests (Denka Seiken, Tokyo, Japan) with and without boiling. The detection limit of the method was <10 cfu/25 g for raw sludge, cured compost and raw compost (for validation data, see Appendix. 3).

Thermophilic *Campylobacter* spp. were analysed according to NMKL 119 (1990) as modified by Lilja (2000). In brief, samples of raw sludge (25 g) were enriched in 225 ml of Bolton enrichment broth (Oxoid) at 41.5°C for 24-48 h. A loopful (10 µl) of the enrichment culture was streaked on mCCDA (modified Campylobacter Charcoal Deoxycholate Agar) plates (Oxoid) after both 24 and 48 h of enrichment and was incubated microaerophilically at 41.5°C for 48 h. Confirmation of typical colonies was performed according to NMKL 119.

3.3.3 Pathogenic viruses

The viral testing for **NLV caliciviruses** and **astroviruses** was performed by RT-PCR; only one of the three samples at each time point was analysed. The samples were prepared as follows. Liquid/semiliquid samples: A 100 µl supernatant sample was used for RNA extraction after centrifugation of the liquid samples at 10,000 g for 2 min. Solid samples: 15 ml sterile distilled water was added to a sample of about 10 g (5.8-21.94 g). The sample was shaken (about 500 rpm) for 1 h at RT, and was thereafter stored at 4°C overnight. A liquid sample was withdrawn and processed as described above. For quantitative determinations ten-fold dilutions were made in distilled water. A 100 µl supernatant was used for RNA extraction.

Two methods for RNA extraction were used: the commercial Tripure extraction was used for the liquid/semiliquid samples and glass milk extraction for the solid samples (Lees et al. 1994), since the latter was more efficient at removing inhibitors to the RT-PCR. In brief, the Tripure extraction is based on phenol-chloroform phase extraction in the presence of guanidineisothiocyanate. In glass milk extraction, nucleic acids bind to glass milk and are separated from inhibitors by repeated washing. Nucleic acids were precipitated by ethanol after both procedures. A part of the NLV polymerase gene region (ORF1) was amplified. The RT-PCR was performed as two separate reactions for genogroups I and II, respectively, as described (Maunula et al. 1999). The primers of Mitchell (Mitchell et al. 1995) were used in astrovirus RT-PCR. The PCR product was confirmed against a single probe (oligonucleotides 6742-62) in a microplate hybridisation assay.

A sample of dry composted organic garden material containing no human waste was used as a control the methods used. Aqueous NLV-containing solutions (concentrations 10^4 and 10^2 PCR-units/ml) were mixed at a ratio of 1.5 to 1 (w/w), were shaken well and were incubated overnight at 4°C in a 50 ml tube. A 2 ml sample from the liquid phase was separated and centrifuged at 10,000 g for 2 min, and 10-fold dilutions for RT-PCR were made in distilled water. The results showed that when higher concentrations of virus were used, there was no measurable decrease in the concentration. An inhibitory effect on the PCR-reaction of the sample up to 1:10 dilutions was observed. For the dilute sample (concentration 10^2 PCR-units/ml) no virus was detected after the incubation. The observed inhibitory effect of the compost sample inhibited the detection of the virus in this case. In conclusion, it appears that the virus is not bound

to the solids of the composted material in any considerable proportion.

3.3.4 Pathogenic protozoa

One gram portion of each of the three well-mixed raw sludge samples collected during three subsequent days from each of the participating sewage plants was taken for concentration and purification of cysts and oocysts by immunomagnetic separation technique (IMS, Rimhanen-Finne et al. 2001) using Dynabeads GC-Combo (Dynal, Oslo, Norway). PCR amplification was undertaken by using primers Cry9/Cry15 to amplify a 550-bp fragment specific for the *Cryptosporidium* oocyst wall-protein (Spano et al.1997) and primers Gdh1/Gdh4 for a 768-bp fragment specific for the glutamate dehydrogenase gene of *Giardia* (Homan et al. 1998). For the genotyping of samples positive for *C. parvum,* the PCR products were digested with *Rsa*I at 37°C for 3 h. The digested PCR products were run on 3% agarose gel and the lengths of produced fragments were measured and compared with those of the *C. parvum* control strain (genotype 2; Spano et al.1997).

Immunofluorescence microscopy was used for the detection of (oo)cysts from composted, cured and other solid samples. In our preliminary tests with inoculated samples, no PCR amplification was seen in any of the solid samples with inoculation levels of 1.25 to 125,000. For microscopic examination of the samples, three aliquots from each well-mixed solid sample were spread into wells on microscopic slides (Waterborne Inc, USA) and the weight of the sample was recorded. After the drying and fixation of the samples, the cysts and oocysts were stained with combined antibodies against *C. parvum* oocysts and *G. intestinalis* cysts conjugated with FITC (Waterborne Inc). The slides were incubated in a moist chamber for 30 min at 37°C, were washed with PBS (pH 7.3) and were examined under an epifluorescence microscope with magnifications of 200x and 400x. The whole area of the microscopic wells was examined, and typically stained cysts and oocysts were counted. Positive controls were always included. Control oocysts and cysts o*f C. parvum* (Iowa isolate) and *G. intestinalis* (H3 isolate) were supplied by Waterborne Inc. (New Orleans, USA) as purified suspensions in an antibiotic solution.

4 RESULTS

4.1 Chemical and physical characteristics

The chemical and physical characteristics studied, i.e. dry and organic matter content, pH and conductivity of sewage sludge samples taken from 22 sewage water treatment plants, peat-sludge mixtures, stabilised sludge, and in the sludge composts of different ages are presented in Table 2 (Appendix 1).

At the windrow composting plants (13-16) the practice was not to measure temperatures from the windrows during the composting process. Thus the temperature data presented are mostly from the time of sampling (Table 2 and Appendix 2). At plant 13 the temperature in the windrow was approximately 77°C at 10 weeks, and it remained high, still over 70°C, at 23 weeks. At plant 14 the windrow temperature was near 50°C at 10 and 30 weeks, and at plant 15 it was near ambient at all times. Temperatures measured during in-vessel composting at plants 17-20 and plants 23-24 are presented in Appendix 2.

4.2 Indicator bacteria

Counts (cfu/g_{dw}) of *E. coli,* enterococci, *C. perfringens* and sulphite-reducing clostridia as well as the presence of *Salmonella,* in sewage sludge samples are presented in Table 3. All these indicator bacteria were present in all the raw sludge samples, except the sample from plant 20, where no *Salmonella* was detected. The contents of the indicator bacteria in raw sludge varied widely; the widest variation was detected in the counts of *C. perfringens,* ranging from less than 100 to over 10^6cfu/g_{dw}. From the raw sludge samples, a total of 22 different *Salmonella* serotypes were isolated (Tables 3 and 4). The antimicrobial susceptibility of these differed widely, and they included four strains (12% of the 33 strains detected) with multiple resistance to the antibiotics tested (Table 4). Only one of the 100 enterococci strains isolated from raw sludge (plant 6) was resistant to both ampicillin and vancomysin; all the other strains were sensitive to both antibiotics.

Salmonella was still found in all of the plants studied after mesophilic anaerobic digestion (plants 1-4). Even a *S.* Haifa strain with resistance to five antibiotics was found in the digested sludge from plant 2. Counts of enterococci and *E. coli* also remained high in these samples. Anaerobic digestion seemed to favour the growth of sulphite-reducing clostridia, which are anaerobic bacteria and thus able to grow without oxygen. Their counts increased slightly during digestion. However, the counts of *C. perfringens* increased at all the digestion plants from ten-fold to ten thousand-fold compared with the levels in raw sludge samples. After composting in windrows with preceding anaerobic digestion, the counts of enterococci and *E. coli* decreased to close to the detection limit at all the composting plants, an ampicillin-resistant enterococcus strain was found in a 10-week-old compost at plant 2. Counts of sulphite-reducing clostridia (plants 1-4) and *C. perfringens* (plants 1-2) remained high. After thermal drying of the digested sludge (plant 4) all the indicator bacteria concentrations were below detection limits, except those of sulphite-reducing clostridia.

After sanitation at in-vessel composting plants (plants 17-20 tunnel composting, plants 21-24 drum composting) *Salmonella* was not detected in the outfeed compost samples, not even at the plants with very short processing times (plants 17, 19, 22). No regrowth of salmonella was found during further windrow composting. However, the enterococci contents were high after tunnel processing at all the plants, although at the same time the sulphite-reducing clostridia contents (plants 18, 19, 20) and also, at one plant (18) the *E. coli* contents decreased. Exceptionally, at plant 17 the counts of all indicator bacteria remained close to those found in raw sludge samples. During curing in windrows after tunnel composting, the enterococci and *E. coli* contents still

remained high at two of the plants (17, 19). Sulphite-reducing clostridia were below the detection limit in all the outfeeds (plants 17-20). *C. perfringens* counts also decreased to below the detection limit in all the samples studied (plants 18, 19) of the 30-week-old composts (Table 3)

High numbers of clostridia were found in the outfeed composts at two of the drum composting plants (21, 23), but at the same time these composts were free of *E. coli* and one also lacked enterococci. The clostridia contents were below the detection limit in two other outfeed composts (22 and 24), but one of them (22) still had a high enterococci content. All indicator bacteria were below the detection limit in the outfeed of plant 24. Two vancomysin-resistant enterococci strains were detected in a sample of a 10-week-old windrow compost from plant 21. During curing in windrows after the drum composting process, enterococci and *E. coli* counts also decreased to below the detection limit in all the composts.

At plants (13-16) where no sanitation process was in use but the sludge was mixed with bark, sometimes also with peat or wood chips, and was composted immediately in open windrows, the counts of clostridia increased during the process. Salmonella was found at plant 16 in samples of 10-week-old compost. However, concentrations of enterococci and *E. coli* decreased after 30 days of composting at all of these four plants, and salmonella was no longer detectable after this stage of the treatment process.

Mixing sludge with peat, without a composting treatment, (plants 9 and 10) had virtually no effect on the counts of the indicator bacteria studied. At plant 9 the numbers of clostridia and *C. perfringens* remained high, whereas the counts of enterococci and *E. coli* decreased slightly. Salmonella was not detected at this plant after the sludge was mixed with peat, but at plant 10 the same multidrug-resistant *S.* Senftenberg as had been found earlier in the raw sludge was found in the sludge sampled after mixing with peat.

Lime stabilisation (plants 5-8) had no sanitation effect at two of the plants (plants 5 and 8). For instance, the same salmonella serotypes (*S.* Virchow at plant 5 and *S.* Senftenberg at plant 8) as had previously been found in the raw sludge samples from the same plants were found in the limed sludges. However, at plants 6 and 7, where the pH in the lime-treated sludge increased noticeably, up to pH 11-12 (see Table 2), the counts of clostridia, enterococci and *E. coli* decreased, the latter two even to below the detection limit. The increased pH also had an effect on salmonella, which was not detected in the treated sludge of these two plants.

4.3 Pathogenic bacteria

Campylobacter was not detected in any of the raw sludges studied. Also, no **STEC** strains were found in any of the raw sludges or in the sludge product samples.

Listeria monocytogenes was found in all the raw sludge samples. After anaerobic digestion, only one of the four sludge samples was positive, and even this turned negative during composting at 10 and 30 weeks (Table 5 in Appendix 1). *L. monocytogenes* was not found in the digested sludge samples after thermal drying. Lime stabilisation did not destroy *L. monocytogenes*, nor did mechanical mixing with peat. The samples from windrow or tunnel composts were all negative, at least at 30 weeks. *L. monocytogenes* was most effectively destroyed in the drum composting systems; all the four outfeed composts were negative, and all the samples taken from the further composted mass were also negative (plants 21-24).

Mycobacteria were not studied in the raw sludge or digested sludge samples. They were found in both of the sludge-peat mixtures (plants 9 and 10) and were recovered from two limed sludge products (plants 5 and 6), but not from any others. Mycobacteria were found in all the compost samples tested, except in the sample with an overgrowth of fungi (plant 17). Mycobacteria were more common in the peat-containing samples than in the other composts (Table 5). No mycobacteria were found in the sample taken from the sludge after the thermal drying process (plant 4).

Performance of the MicroSeq 500 assay for

the identification of *Mycobacterium* isolates: A total of 15 isolates were subjected to 16S-rDNA-sequence analysis by the MicroSeq 500 assay. For ten of these, the results of the original identification were concordant with the results of the MicroSeq identification, and five isolates had a molecular identification that was discordant with the original identification. Isolates were grouped into *M. terrae*/*M. nonchromogenicum* (Table 5).

4.4 Pathogenic viruses

The presence and survival of the two genera of enteric viruses was studied: the NLV-**caliciviruses** (**Noroviruses**) and human **astroviruses** (Table 5). NLVs were present in practically all of the samples at the start of the follow-up period, though the concentrations varied to a large extent (from 1 to >1000 PCR units/100µl sludge), apparently reflecting the epidemiological situation in the respective populations. In Finnland more NLV outbreaks are caused by NLV genogroup (gg) II than I (Maunula et al. 1999). As a logical consequence of this, the concentration of ggII exceeded that of ggI in all except one sample in the present study. The astroviruses that were found in the samples and their counts varied probably according to the epidemiological situation.

After anaerobic digestion (plants 1-4) NLVs were found in all the four plants, and also astroviruses in both the plants examined. After windrow composting of the digested sludge, however, neither calici- nor astroviruses were found. Lime stabilisation of the sludge was effective enough to kill both viruses at two plants (6 and 7) but not at the others two (5 and 8). The positivity was also retained after 10 weeks of treatment at two of those sludge treatment plants (15 and 16) where windrow composting was the only sanitation method. However, after 30 weeks of treatment all the samples from the composted sludge were negative for both NLVs and astroviruses (plants 13-16). Outfeeds from tunnel composting systems were mostly negative (18, 19 and 20), only from plant 17 were both NLVs and astroviruses detected. NLVs were found in all outfeeds from drum composting systems, but not after curing for 10 weeks. When the sludge was mixed with peat without composting (plants 9 and 10), no viral sanitation was observed.

4.5 Pathogenic protozoa

Giardia was found in the raw sludge from 14 of the 22 plants studied and *Cryptosporidium* in the raw sludge from 20 plants. The analysis of three subsequent sludge samples from three subsequent days revealed that a positive plant either had only one positive sample or all three subsequent samples were positive. Variation was seen between various sludge treatment processes in the efficiency of destroying cysts and oocysts. After anaerobic digestion, *Cryptosporidium* was found in the sludge samples from two (1 and 2) of the four plants, but no *Giardia* was found. After the composting of digested sludge in open windrows, *Giardia* was found in all the composts of 10 and 30 weeks of age (1-4). However, all these samples were negative for *Cryptosporidium* oocysts. Samples from four plants using windrow composting as the only sanitation method (plants 13-16) were positive for both giardia and cryptosporidia at both 10 and 30 weeks. After lime stabilisation (plants 5-8), only cysts of *Giardia* but no oocysts of *Cryptosporidia* were found. Lime stabilisation was efficient at two of the plants, where the pH of the sludge at the time of sampling was about 11. Cysts were present in the sludge with lower alkalinity (plant 8). When sludge was only mixed with peat, both cysts and oocysts were detected in all these sludge products (plants 10 and 11). When sludge was cured in windrows after drum composting, oocysts (plants 21, 22 and 24) and cysts (22 and 24) were found at 10 weeks, but at 30 weeks only cysts were found at one of the plants (22); and after 10 weeks of curing, tunnel composted sludges were negative for cysts, but oocysts were detected at one of the plants (20). At 30 weeks, neither cysts nor oocysts were detected in any of these composts. Thus the most efficient procedures were drum and tunnel composting systems followed by 30 weeks curing in windrows. Open windrow or mattress composts were less efficient.

5 DISCUSSION

5.1 Behaviour of pathogenic and indicator microbes in various sludge treatment processes

Raw sewage sludge (Plants 1-10, 12-24)

The wastewater plants selected for this study treated sewage water of approximately 1.8 - 2 million of the total 5.2 million inhabitants in Finland. None of the plants had a slaughterhouse wastewater intake, and thus most of the load of intestinal pathogens was of human origin. The microbes monitored in this study are either human enteric pathogens (*Salmonell*a, NLV-viruses, *Giardia*, *and Cryptosporidium*) or they belong to the normal flora of the human intestine (*E. coli*, enterococci, and clostridia) and are released into sewage at high concentrations. Clostridia and mycobacteria belong to the normal microbiota of soil and related material, but some, e.g. clostridia species (*C. perfringens*) are also part of the normal flora of the human and the animal intestine. Most of the microbes monitored were detected in all the raw sewage sludge samples.

The nature and concentration of pathogens in sewage, and hence in sewage sludge, depends on the health status and size of the population in the catchment area. This was also seen in our results from 22 wastewater treatment plants in different geographical locations in Finland and with wide variation in the size of the population they served. For instance, variation in *Salmonella* serotypes and the presence of viral pathogens was detected. The presence of NLV caliciviruses in all the raw sludge samples is probably explained by their typical epidemics in early spring, which was when our sampling of raw sludge was carried out. However, *Salmonella* and *L. monocytogenes* are excreted without any major outbreaks, because they were found in the raw sludge at almost every plant. Only a small STEC cluster occurred during spring 2001, and no STEC was detected in any of the samples. In France, Vernozy-Rozand et al. (2002) found 53% of the sewage sludge samples sxt-positive with PCR, but they had no data available on human isolations in the area. The absence of campylobacter positive raw sludge samples may reflect a problem of sampling and/or death of bacteria during transportation, because patients excreting campylobacteria existed also in April 2001 in Finland (277 campylobacter cases reported from whole country to NIRD in April).

Viral pathogens other than NLVs may also occur at high concentrations in sewage, but their circulation in the aqueous environment through waterborne outbreaks has not been documented in industrialised countries. One exception is astroviruses, which have been found in sewage (v. Bonsdorff et al. 2002b) and were recently also detected as causing a waterborne outbreak related to a children's swimming pool in Finland (Pönkä et al. 2002). On the other hand, numerous HAV outbreaks caused by sewage-contaminated shellfish have been reported (for references, see Lees 2000).

Our results confirmed the earlier results from spring 2000 (MMM publication 2/2001) that *Giardia* cysts and *Cryptosporidium* oocysts are more common at Finnish municipal sewage plants than estimated on the basis of reported human cases in Finland (NIDR). Reported cases have varied annually from 10 to 20 for *Cryptosporidium* and from 250 to 300 for *Giardia*. Medema (1999) studied the discharge from Dutch sewage plants into the Rhine and Maas rivers and found that all their samples contained *Giardia* and *Cryptosporidium*, further suggesting that excretion of these organisms is common in western European countries. In municipal raw sludge, the concentration of *Giardia* cysts is usually higher than that of oocysts of *Cryptosporidium*; this reflects differences in the numbers of people excreting these pathogens (Medema 1999, Robertson et al. 2000, de Wit et al. 2001). Genotyping was performed for PCR products of *Cryptosporidium* only, and these results showed that all positive samples were of genotype 2. This genotype is a zoonotic genotype infecting both humans and ruminants (Spano et al. 1997, Slifko et al. 2000).

Stabilisation by lime treatment (Plants 5-8)

Strauch (1998) has reported that the combination of pH 12 and temperatures in the range of 60 to 70°C – typical when quicklime is mixed with sludge – destroyed *Ascaris* ova and some viruses within 24 hours. Lime treatment of raw sludge at pH 12.8 is also known to destroy *Salmonella* Senftenberg completely within 3 hours (Shaban 1999). Carrington (2001) thus recommended that, in order to produce a pathogen-free sludge product, the sludge and quicklime should be thoroughly mixed, the pH should reach 12 and the temperature should be 55°C for fewer than two hours.

In our study, most of the pathogens, including *Salmonella*, were not detected and the counts of all the indicator bacteria decreased in the lime-stabilised sludge samples when the pH measured in these samples was over 11 at the time of sampling. In practice, however, mixing the sludge with lime may sometimes be inadequate, because the lime-stabilisation process was not always sufficient to destroy pathogens or indicator bacteria, as shown in the present study, as well as in Straub et al. 1993 and Rimhanen-Finne et al. 2001.When the pH remained near neutral, the same salmonella serotypes were found in the limed samples as in the raw sludge (*S*. Virchow at plant 5 and *S*. Senftenberg at plant 8); no changes in the counts of indicator bacteria were detected.

Stabilisation by anaerobic digestion (Plants 1-4)

In Finland, mesophilic digestion processes are in use for sludge stabilisation treatment only. Mesophilic anaerobic digestion operated at temperatures around 35°C, an optimum growth temperature for most of the enteric bacteria, can be deleterious for the survival of pathogens, because the process produces fatty acids and other antigenic products in amounts that are lethal to many pathogenic organisms (Carrington 2001). However, our results show that a 21-day mesophilic digestion was not effective enough to kill pathogens. *Salmonella*, calici- and astroviruses, as well as cysts and oocysts of protozoa, were found in all digested sludge samples, and *L. monocytogenes* at one of the plants. Anaerobic digestion seemed to favour the growth of both *C. perfringens* and other sulphite-reducing clostridia, which in fact are essential for the process to function properly. Furthermore, Carington (2001) has stated that the production of a sludge virtually free of pathogens is not possible with mesophilic systems alone. Thus he recommends that the sludge should be treated with moist heat at 70°C for 30 minutes prior to immediate mesophilic anaerobic digestion. According to our results, composting significantly decreased enterococci contents, and no *Salmonella*, NLVs, astroviruses or *L. monocytogenes* were found in digested sludges after open-air composting. However, composting was not capable of decreasing clostridia counts significantly. *Giardia* were also detected from digested sludge after composting. On the basis of our results, in order to obtain a sludge product with a low level of pathogens after anaerobic digestion the sludge has to be further treated either by thermal drying (as at plant 4) or by adequate composting (as at plants 1-4).

Thermal drying (Plant 4)

Carrington (2001) recommends that when the sludge is to be thermally dried its temperature should be raised to 80°C for ten minutes and its moisture content should be reduced to less than 10%. In the present study only one plant used thermal drying (90°C, dry matter content 9%) as a sludge treatment method after mesophilic anaerobic digestion. After the treatment, no pathogens were detected, and the counts of the indicator bacteria decreased. Only sulphite-reducing clostridia producing highly heat-resistant spores were found at approximately 3000 cfu/g_{dw}. Clostridia are commonly found in the environment, i.e. in soil, and most of them do not represent a health hazard, but some might. This finding regarding clostridia indicates that organisms producing heat-resistant structures are able to survive this kind of treatment.

Sludge treatment by mechanical mixing with peat (Plants 9 and 10)

We have no knowledge of any recommendations for mechanical mixing with peat as a sludge treatment method. Only two plants in our study used mixing with peat as the sole treatment prior to

agricultural use of the sludge. At one of the plants the counts of enterococci and *E. coli* decreased and salmonella was not found in the mixture; however, at the other plant the counts of all the indicator bacteria were unchanged and the same salmonella serotype (*S*. Senftenberg) as was present in the raw sludge was detected again. Even if the data are too limited for the drawing of any conclusions, NLVs, *Listeria*, protozoa and *Salmonella* were found in the end products, indicating that these products are not safe for uses in which animals or edible plants are in close contact with the product. Mycobacteria were also found in both of the peat mixtures.

The number of *Mycobacteria* recovered was higher in the peat-containing samples than in the other treated sludge products. This suggests that *Mycobacterium* may benefit from acidity in the growth media or they may originate in peat. *Mycobacteria* were also found in peat used for mixtures or as bulking agents in composting. Peats are rich in environmental mycobacteria, and the acidity of peat may even stimulate their growth (Iivanainen et al. 1997).

Composting (Plants 13-24 and plants 1-4 after mesophilic digestion)

Composting is a microbial, aerobic process involving dynamic changes in temperature, oxygen concentration, moisture content, nutrient availability and organic matter evolution, as well as in the microbial population. It can be technically divided into two phases: active composting in in-vessel composting system and further composting of the raw compost in open windrows. This latter phase is also called curing. It is recommended that composting should be carried out in batches (tunnel composting systems or windrows) or in "plug-flow" systems (i. e. drum composting) over a period sufficient to stabilise the sludge and to produce an acceptable product (Carrigton 2001). All material should be maintained at a temperature of no lower than 55°C for at least 4 hours between turnings; the number of turnings in a windrow system should be at least three. It has also been stated that the temperature of the composting material should be at 55-65°C for 1 to 3 days in order to achieve hygienisation (Déportes et al 1998). Stenström (1996, in Johansson 1997) has proposed that there is no risk of the survival of *Giardia, Cryptosporidium, Toxoplasma, Campylobacter, Salmonella, E. coli* and *L. monocytogenes* when the temperature exceeds 60°C for at least two days during the composting process, followed by a maturing period. Our results show that not only does a high temperature kill the pathogens but cold and anaerobic conditions may also, with sufficient time, sanitise the sludge. Plant 15 is an example of this, with poor composting (low process temperatures), and at plant 2 the digested sludge was aged in a low mattress without any addition of bulking agent or turning. At these plants the levels of indicator bacteria decreased and pathogens disappeared, but counts of clostridia remained high in the sludge products at 30 weeks. Also Déportes et al. (1998) have reported that sanitation is possible in time at low temperatures.

Reasons for poor sanitation in in-vessel composting systems may be diverse. An undersized system causes the composting mass to remain in the process for too short time, and too high a rate of filling prevents the flow of air through the composting mass. The main reason for poor composting and sanitation in in-vessel composting systems is probably the lack of oxygen. That was, as expected, situation at one of the tunnel composting plants (17) with only a six-day delay time. High levels of anaerobic sulphite-reducing clostridia and *C. perfringens* were detected in the outfeed compost of this plant, suggesting a low oxygen content during the process.

Two of the drum composting plants (21 and 22) were also known to have some technical problems with aeration during our study. Thus, poor sanitation was expected, but plant 22, with a delay time of only four days at 45-50°C, managed to free the outfeed of clostridia, *L. monosytogenes* and *Salmonella*. *E. coli* counts were also low. Enterococcal counts decreased at two of the drum composting plants (21 and 24), but remained quite stable at the other two (22 and 23), without any association with technical problems.

Well performed and adequate further com-

posting or curing, for instance in open windrows or mattresses, is necessary in order to obtain a good-quality compost product. Curing may also improve poor in-vessel composting and the sanitation state of the compost, as seen in this study. In contrast, Droffner & Brinton (1995) have shown that *E. coli* and *Salmonella* were present in biowaste compost at 59 days. The biowaste was first composted in indoor channel-windrows for fifteen days and further in piles at 59°C for 44 days. In laboratory trials Droffner & Brinton (1995) showed that in a sludge compost *E. coli* and *Salmonella* remained cultivable for at least 11 days. If relevant time-temperature characteristics are achieved, i.e., the temperature during composting is at least 55°C for four hours between turnings (Carrington 2001), pathogens are destroyed. The efficiency of turning is of crucial importance for the temperature to reach 55°C in all parts of the compost. Measuring the moisture content (a_w), another physical parameter, during composting might generate more useful information, because many organisms survive better in high a_w than in low a_w.

Survival of potential pathogenic microbes in composted sewage sludge depends on time, temperature, pH, oxidation-reduction potential, moisture, nutrient supply, organic matter, dry matter content and indigenous microorganisms. A major reason for enteric bacterial die-off outside of the host intestinal tract is probably their inability to lower their metabolic requirements in response to a lower nutrient availability. The organic contents of sludge enhance bacterial survival. It is uncertain which inactivation mechanisms are involved. Pathogens may reside in the cooler zones of windrows or as clumps along the edges of static piles. A rapid dehydration process may cause increased microbial inactivation, but long-term survival is improved by low water activity. Intra-protozoal growth of bacterial pathogens has been associated with increased environmental survival, virulence and resistance to biocides and antibiotics. Using laboratory microcosms it has been shown that STEC O157 and *L. monocytogenes* survived and replicated in a common environmental protozoan, *Acanthamoeba polyphaga,* in experiments in vitro (Barker et al. 1999, Walcher et al. 2001). Other bacterial pathogens that may multiply or survive in amoebae include opportunist mycobacteria and coliforms. Since protozoa are widely distributed in soil and sewage, they may assist the survival of pathogens through the sewage treatment process (Barker et al.1999).

In our study no regrowth of pathogens was detected during composting. On the other hand, natural regrowth of *E. coli* and *Salmonella* in buried faeces and in municipal solid waste compost has been detected (Déportes et al. 1998). Both thermotolerant and mesothermophilic *E. coli, S.* Typhimurium and *P. aeruginosa* mutant, able to grow at temperatures up to 54°C or more, have been isolated (Droffner & Yamamoto 1992), and there is evidence that under certain conditions even *Salmonella* strains are able to mutateso as to grow at 68°C (Brinton & Droffner 1994). Microbial regrowth potential is affected by a number of different inherited and environmental factors; thus the possible regrowth of pathogens is difficult to predict. For instance, *Salmonella* was affected by moisture, temperature, indigenous microbial competition and the nutritional status of compost (Sidhu et al. 1999). Sidhu et al. (1999) proposed that a pathogen regrowth potential test could be used for ensuring the bio-safety of composted biosolids products prior to their being placed on the market . Also Déportes et al. (1998) stated that it is important to view the microbiological quality after storage because of the risk of natural regrowth and contamination during storage.

5.2 Reliability of the results

The results of our study are mainly in agreement with those of our pilot experiment (MMM publication 2/2001). However, the hygienic quality of the sludge after anaerobic mesophilic digestion was lower in the present study than in the pilot experiment.

Some of the pathogens monitored in this study, such as viruses or protozoa, were not always found in the initial material, i.e. the raw sludge samples from the wastewater treatment plants. However, they were detected in successive samplings from the same plants. This suggests that the pathogens were probably unevenly distributed in the successive raw sludge samples, or that contamination occurred during the sampling or sludge processing, the latter two being less plausible.

The results of the validation study show that *L. monocytogenes* was more difficult to detect in the raw sludge and outfeed samples (detection limit 30 cfu/25 g) than in the samples of compost at 30 weeks (detection limit 3 cfu/25 g). Competitive flora, especially overgrowth by *Bacillus* species, interfered with the detection on both improved LMBA and PALCAM medium. *L. monocytogenes* was most often detected on improved LMBA after half-Fraser enrichment. During Fraser enrichment, overgrowth by other *Listeria* species occurred and *L. monocytogenes* was difficult to isolate on PALCAM media, which is unable to differentiate *L. monocytogenes* from the other *Listeria* species. Johansson (1998) previously reported similar results in the analysis of foods and environmental samples. It would have been interesting to obtain data on the levels of *L. monocytogenes* in sludge and sludge products. However, according to the preliminary study (data not shown) enumeration could not have been reliably performed using direct plating on improved LMBA or PALCAM media, owing to abundant competitive bacteria. However, *L. monocytogenes* was detected by enrichment in all the raw sludge samples and thus contained *L. monocytogenes* in counts above 1.2 cfu/g.

According to the results of the validation study (Appendix 3), the detection limit of the culturing method for STEC O157 was low (9 cfu/25 g, equal to 0.4 cfu/g) in the analyses of raw sludge and outfeed samples with high microbial loads. However, *Pseudomonas* spp. cannot be eliminated from the enrichment culture by immunomagnetic separation, and this might particularly cause failures in the detection. *Pseudomonas* was problematic because of its colony morphology similar to that of STEC O157 on the selective plates, and it especially appeared on SMAC plates after a 24-h enrichment period. In the validation study with vital STEC O157 cells, the enrichment period of 6 h proved to be superior to 24 h, and CT-SMAC superior to SMAC. Lahti et al. (2002b) also reported more positive results with a 6-h enrichment period than with 24 h in the analysis of STEC O157 in environmental samples taken from farms, including e.g. samples from the floors of pens. However, *E. coli* O157 is easily stressed and therefore the enrichment period of 24 h in addition to 6 h, and SMAC in addition to CT-SMAC, were used in the analysis of sludge samples.

It is obvious that the levels of STEC in sludge were very low, because its incidence in humans in Finland has been very low. Even the highly sensitive culturing method for the detection of STEC O157 was incapable of detecting any positive samples. It is probable that even lower levels, lower than 5 cfu/25 g, could be detected in sludge; such levels were detected in bovine faeces, in regular analyses of inoculated control samples of bovine faeces during several years by using the described method. In contrast, the PCR procedures commonly used for the analysis of STEC from bacterial cultures of human clinical samples might be inappropriate for the analysis of sludge. The PCR performed on the direct culture on SMAC was as sensitive as it could theoretically be (detected 10^3 - 10^4 cfu/g), when 1.5 µl taken from the suspension containing a loopful of the SMAC culture suspended in 500 µl of sterile water was analysed (the assumption being that 10 ml of sludge was equal to 30-80 mg). However, it is unrealistic that the levels of STEC in sludge would be as high as 10^3 - 10^4 cfu/g.

The PCR performed on an enrichment culture might also not be suitable for the analysis of STEC in sludge with probably abundant inhibitory substances and overwhelming counts of competing microbes. Elimination of inhibitory substances is essential for successful performance of PCR when enrichment cultures are used, and it needs improvement. Commercial products are currently available for this purpose, and they have been successfully applied to analyses of sludge samples

(Vernozy-Roland et al. 2002). The performance of the PCR method could be further improved by increasing the selectivity of novobiosine containing mTSB by incubating it at 41.5°C instead of 37°C. STEC O157 is known to be more heat-sensitive than is indicator *E. coli*. Poor growth of STEC O157 strains has been observed at temperatures above 42°C (Raghubeer et al. 1990). Therefore, incubation at 37°C was used for enrichment in the PCR procedure. However, enrichment of STEC O157 strains at 41-42°C was considered preferable (Raghubeer et al. 1990). The enrichment temperature of 41.5°C was adopted by both ISO (ISO 2001) and NMKL (NMKL 1999), because the procedures described in these methods were shown to be the most sensitive and specific (Bolton et al. 1996; de Boer & Heuvelink 2000; Scotter et al. 2000). CT-SMAC should be used in addition to SMAC in the PCR procedure as well, in order to detect both STEC O157 and non-O157 strains. CT-SMAC is more optimal than SMAC. However, it has been shown to be inhibitory to some STEC O157, as well as to some non-O157 strains (Aleksic et al. 1992, Laaksonen 1999). An enrichment period of six hours could have increased the sensitivity of PCR and might be worth testing in the future. Furthermore, a drawback of PCR is the difficulty of identifying a STEC colony from a selective plate for further typing. Fishing out a STEC colony from the culture is usually laborious and requires tens and even hundreds of colonies to be tested once more by PCR. Unfortunately, no standardised culturing method for the detection of non-O157 STEC exists so far, and the method is difficult to develop because of the similar biochemical characteristics of this group and the indicator *E. coli* group.

Vernozy-Rozand (2002) detected as high an amount as 34% of the effluent samples from sewage wastewater plants (n=437) to be stx-positive by PCR. However, the authors suspected that the presence of bacteriophages in sewage carrying stx genes could give positive results from stx PCR reactions, causing an overestimation of STEC. The authors suspected that this might also explain the low number (26%) of STEC strains they were able to isolate from the stx positive enrichment broths by colony hybridisation in the analyses of the samples from wastewater treatment plants and waste storage lagoons. In addition, the assumption was supported by the fact that the proportion of positive PCR was significantly greater in municipal wastes than in farm wastes. Muniesa and Jofre (1998) reported in urban sewage high levels of stx2 gene carrying phages, which remained infectious and were able to infect *E. coli* O157 strains. Vernozy-Rozand et al. (2002) were able to detect only one *E. coli* O157: H7 strain among the PCR positive samples.

In general, PCR methods are suitable for pathogen monitoring in sludge in controlled conditions when used in combination with other microbial tests. However, for reliable determination of the detection limits of the PCR, validation of the sewage matrix with a consistency equivalent to that of the sample to be studied is required.

The amplification of parts of microbial genes (e.g. PCR or RT-PCR) is a sensitive method for showing the presence of microbes in samples. For some microbes, such as human NLVs, it is at present the only method, which can catch all the known different virus strains. The counts of infective viruses or other microbes in sewage sludge cannot, however, be shown by PCR methods. The PCR positivity seen after lime treatment possibly does not reflect infectivity, since a pH above 10 is known to inactivate practically all viruses (Knipe & Howley 2001). With poliovirus and bacteriophage, Sobsey et al. (1998) showed that PCR assays underestimated the disinfection of viruses with UV radiation and free chlorine when the time scale was short (max 30 min). During the long processing time used for sludge samples the inactivated virus particles will break down, the exposed genomes will rapidly be destroyed and the virus can no more be detected by PCR.

The main concern is the proper sample handling for PCR. From the liquid phase of sewage the nucleic acids can be extracted reliably, but for more solid samples it is difficult to ensure the detachment of microbes. Our preliminary results suggested that, when the amount of virus was high, the viral nucleic acid had been gathered efficiently, but when the amount was low, all virus was lost.

More investigations are needed to improve sample handling. Since the sludge is not homogeneous, sludge samples with various compositions should be included in the analysis.

Immunofluorescense microscopy has earlier been used for the detection of protozoal cysts and oocysts from sewage samples (Major & Palmer 1996, Medema 1999). In the present study, we used immunofluorescence for samples of different kinds of processed sludge. Microscopic studies revealed that cysts and oocysts would survived in various sludge treatment processes. One of the disadvantages of both PCR for protozoa and microscopic techniques are that they do not distinguish between viable and nonviable cysts or oocysts. For safety assessment, more studies where viability studies are included will be needed. The IC- PCR technique did not work for solid samples when tested with inoculated samples. Either IMS is not strong enough to capture cysts and oocysts or the composted material had compounds inhibiting PCR reaction.

5.3 Indicators of the hygienic state of sludge products such as composts

The discussion, based on our results about the suitability of the pathogens studied for checking the hygienic safety of a product is limited mostly to composted sludge products.

The monitoring of composts should preferably be non-labour, intensive and inexpensive. The indicator organism should be suitable both for checking the hygienic safety of the product and for evaluating the treatment process for its capability to inactivate pathogens That are of significance for public health. The method should also be sufficiently sensitive because the number of pathogens may change rapidly, depending on the infective status of the population

Compost is a very heterogeneous material, and thus difficult to analyze for microbiological parameters. The changes in their physiological state that bacteria undergo to be able to survive in hostile environments also cause some technical difficulties in their detection. Furthermore, sampling from a large amount of composted material in the field and a large volume of the composite sample transferred to a laboratory for monitoring raise the question of representative sampling and subsampling procedures. In this study, depending on the microbes to be analysed, subsample sizes varied from approximately 100 mg to 25 g. If pathogens are not found, it does not necessarily mean that they are absent in the compost windrow or in the composite sample taken from the windrow, or even absent in the subsample taken for analysis in the laboratory.

The methods used for most of the microbes have not been developed for analysis of waste material; most of the bacteria were studied using methods developed for foodstuffs or animal feeds. Isolation methods using a selective medium without a nonselective first step may not be suitable for isolating bacteria from thermal environments (Droffner & Brinton 1996). On the other hand, a non-selective step may support the growth of unwanted microbes. Overgrowth of faster growing bacteria and fungi may cause problems, as was found with STEC, *Listeria monocytogenes* and mycobacteria in this study. The method validations done for this study showed, however, that the conventional bacteriological culture methods were well suited for our purpose. Because of the overgrowth of faster growing bacteria and fungi, decontamination with oxalic acid requires further modification in order to be applicable to *Mycobacteria* analysis on sludge. All cultivation methods for *Mycobacteria* are all too slow for safety control. For risk assessment and legislation, development of a rapid real-time quantification method for viable mycobacteria is called for.

For reliable assessment of the hygienic quality of a successful sanitation process, indicator organisms whose destruction correlates with that of pathogenic bacteria should be monitored. It is important to combine several indicators or pathogens to assess sanitation (Déportes et al. 1998). *E. coli* and other bacteria of the *Enterobacteriaceae* family have in general a low resistance to heat and the other environmental stress factors. Shaban (1999) showed that faecal streptococci were more resistant to the composting processes in different

kinds of open windrows than were other organisms. Staurch (1998) suggested that, immediately after processing, no *Salmonella* should be detected. According to the USA EPA regulations for "Class A" composts the *E. coli* content should not exceed 1000 cfu/g_{dw} (Anonym 2002). Böhm (2002) widely discussed suitable indicators for compost products and stated that one of the most useful and reliable is the absence or presence of *Salmonella*. Carrington (2001) also suggested *E. coli* and *Clostridium* spp (e.g. *C. perfringens*) as suitable candidates for indicator organisms. He proposed that the levels of *E. coli* should not exceed 1000/g_{dw} and that the maximum concentration of *C. perfringens* spores is 3000/g_{dw}. However, our results show that clostridia are not ideal indicator organisms, because their concentrations in sludge material can increase during digestion or inadequate composting when the oxygen concentration decreases. Clostridia are ubiquitous in nature, and *C. perfringens* is found at 10^3-10^4 cfu/g in soil and 10^3-10^6 cfu/g in human faeces.

No relationship was found between the inactivation of *L. monocytogenes* and the counts of sulphite-reducing clostridia or *C. perfringens*. *L. monocytogenes* was present when the count of enterococci was over 1000 cfu/g_{dw} and the count of *E. coli* over 100 cfu/g_{dw}. When *Salmonella* was not found, *L. monocytogenes* was not always absent.

Salmonella-free sludge products commonly contains *L. monocytogenes*, NVL caliciviruses, astroviruses, *Giardia* and *Cryptosporia,* and thus *Salmonella* alone is not an adequate indicator organism for monitoring the human pathogen risk in sludge products. At least the counts of *E. coli* and enterococci should also be monitored and the results should remain below 100 and 1000 cfu/g_{dw}, respectively. However, according to our results, for instance, the outfeeds from drum composting plants may contain NLV caliciviruses and protozoal cysts even when *Salmonella*, *E. coli* or enterococci are not found.

The NLVs belong to the most resistant human viruses along with hepatitis A virus. Thus they serve as good indicators of safe waste handling. Their presence seemed to have a close relationship with the presence of *Listeria*, except in the drum composting process, which favoured the survival or detection of viruses.

M. terrae is a possible *M. tuberculosis* surrogate for use in clinical disinfection studies. In the future, non-pathogenic *M. terrae* or *M. chromogenicum*, a natural inhabitant in compost, could be used as an indicator of pathogenic mycobacteria in composting processes.

The fate of STEC in the composting process of naturally contaminated sewage sludge remains unclear in this study. Using PCR, Vernozy-Rozand et al. (2002) found in France 16% of sewage sludge samples positive. Odgen et al. (2002) showed that *E. coli* O157 survived in soil for 15 weeks. *E. coli* is not a suitable indicator of STEC, because the growth temperature of the method is too high for STEC. STEC and *E. coli* also have different abilities to resist environmental stresses.

5.4 Risk assessment

Risk assessment is a process engendered because of the need to make risk management decisions in the face of uncertainty. Risk assessment follows a series of steps, including hazard identification, hazard assessment, exposure assessment, risk characterisation and risk assessment, after which risk management is possible. Judging from our results, it is possible to perform the two first steps: hazard identification and assessment; however, only speculation and discussion of exposure assessment is possible.

Pathogens and their toxins are among the primary hazards for sewage sludge treatment workers. Sludge products may also be hazardous to consumers and to the environment if sanitation is poor or the sludge contains potentially pathogenic microorganims the proliferation of which is possible during composting.

Pathogens are most abundant in raw sludge and poorly sanitated sludge products such as sludge-peat mixtures and digested products. Thus, the potential health impact on workers in composting facilities is highest when raw materials

or poorly sanitated products are handled. Exposure is possible in the handling of raw feedstock during transportation and mixing, and from dust and aerosols around the conveyor belts, mixers and other equipment (Epstein 2002).

Many microbes, including pathogens detected in the present study are common in our local environment. Reduction of the infection risk is an important question in the working environment of a composting plant. The pathogens are dangerous only to sensitive people or to those with low resistance on account of weak health. Many microorganisms could be transmitted by inhalation of bioaerosols generated during sewage treatment; however, the health impacts of such bioaerosols on people working at sludge treatment plants have not been reported.

During composting, the numbers thermophilic and thermotolerant microorganisms increase in numbers. Because some of these microorganisms are able to produce spores, release of such propagules into the surrounding air occurs during various steps of the waste treatment process (Kämpfer et al. 2002). Air inversions in particular may lead to high microorganism concentrations (> 10^4- 10^3 cfu/m^3 of thermophilic actinomycetes and fungi) in the surrounding composting plant (Kämpfer & Albrecht 2002). Kämpfer & Albrecht (2002) have discussed widely the risks of bioaerosols.

It is known that actinomycetes and all mycobacteria cause a risk to human and animal health, and they may survive or even multiply in sludge treatment processes or mycobacteria may be present in some bulking agents commonly used in sludge composting. The minimum infective dose of environmental mycobacteria is poorly known, but the most relevant risk groups are the young, the old and people or animals that have an immunosuppressive illness. An infection caused by antibiotic-resistant mycobacteria can be life threatening also for healthy adults and is thus a relevant risk to workers in composting plants.

There are no infectivity measurements available for NLVs, except on human volunteers. These viruses are, however, non-enveloped single-stranded RNA-viruses. If the capsid is damaged, it is highly unlikely that any of the RNA would persist to cause positive RT-PCR results. Thus the obtained positive findings should be considered as indicating an infection risk.

One of the most important aspects in the human or animal health hazard assessment is the viability of unculturable enteric pathogens, such as cysts of *Giardia* and oocysts of *Cryptosporidium* and those of caliciviruses after various treatment processes. Assessment of the viability of cysts or oocysts involves methodological difficulties, because the most reliable viability assay is oral inoculation of susceptible animals and the monitoring of the colonisation capacity of cysts or oocysts. In our studies, no viability testing was performed. However, the presence/absence of visible cysts or oocysts is most probably a marker of the general efficiency of a treatment process in destroying most of the resistant human and animal pathogens.

Salmonellae present in raw sludges were mainly destroyed by sanitation. However, the performance of certain sanitation methods (for instance lime) was poor. Depending on the epidemiological situation, a variable number of salmonella serotypes with different antimicrobial resistance patterns may be present in sludge products for instance, after lime stabilisation. Mixing with peat did not destroy salmonella either. Thus, the use of sludge products for soil fertilisation may pose a salmonella risk to humans and animals. In the literature there are plenty of examples of salmonella outbreaks associated with fresh produce when the fertiliser contained salmonella or many other pathogenic microorganisms (see references in Böhm 2002).

There have not been any reported or documented cases of bacterial, viral or parasitic diseases among workers engaged in composting or among compost users or visitors (Epstein 2002). However, Lewis and his co-workers (2002) in their very recent article pointed out that residents who lived within approximately 1 km of sewage sludge application sites generally complained of irritation, e.g., skin rashes and a burning sensation in the eyes, throat, and lungs, after exposure to winds blowing from treated fields. A prevalence of *Staphylococcus aureus* infections of the skin and the respiratory

tract was found. They reported that approximately 14 of 54 individuals were infected, including two mortalities, indicating that the airborne biosolids may play a role in risks of infections. In Finland, subjects living near a composting facility (Paunio 2002) have complained of similar symptoms.

When air quality was monitored after land application of anaerobically digested sewage sludge, the risk of transmission of *Salmonella* and pathogenic clostridia was found to be low (Pell 1997). The role of environmental *C. perfringens* strains in human food poisonings is not fully understood, but fewer than 5% of the environmental strains are capable of producing enterotoxins. A problem might arise in an epidemic situation, if enterotoxigenic strains became dominant in the digested mass, and the heat-resistant spores survived the composting process.

Sewage sludge or sludge compost should not be applied to agricultural land where vegetables or other crops eaten raw will be cultivated. Even a low level of NLVs or STEC contamination could present a significant health risk to humans, since their infective dose is minimal; even one viral particle or a few bacterial cells may cause an infection. Other pathogens may also become a risk, since the susceptible population and the number of subjects belonging to certain risk groups are increasing globally. Edible portions of plants may be contaminated through transport of the viral or bacterial pathogen into the plant by its root system, without any direct contact with a pathogen (Solomon et al. 2002). Microbes are also known to remain inside a growing seed in certain circumstances (Cambell 1985); this mechanism has been the reason for large salmonella epidemics caused by sprouts both in Finland and in some other countries (USA, England, Canada etc.).

The same *Salmonella* serotypes have been detected from both farm animals and humans in Finland. Even if sludge could theoretically be a source of salmonella, the prevalence of salmonella in domestic animals in Finland is so low that the role of sludge in these salmonella infections is marginal. In general, despite the widespread application of slurry to farmland and its frequent contamination with salmonella, there have been surprisingly few major outbreaks of salmonellosis shown to have resulted from this activity (in Vernozy-Rozand 2002). Perhaps the significant question is not salmonella positivity, but rather the number and the distribution of infectious doses in the material.

If pathogenic bacteria resistant to antibiotics are present in the environment or at the beginning of the food production chain – in the field – they may contaminate the food and feed chains either directly or by transferring their resistance genes to other bacteria, even if the bacteria belong to different ecological niches. Ingestion of food products with antibiotic-resistant bacteria or antibiotic-resistance genes carries strong potential for the spread of resistance (Harrison et al. 1998). The major health implication of resistance development is the danger of treatment failures in humans or animals. Resistant salmonella may have an increased ability to colonise animals or persist in the environment, thereby increasing the risk of food borne salmonella in general (Wegener et al. 1999).

The present study leaves certain questions unsolved and calling for further study: What is the fate of STEC during composting? Will PCR be a rapid, inexpensive and useful tool in the future? If the counts of *Clostridium perfringens* increase during the composting process, could the quality of the product be verified by analysis of the presence of *C. perfingens* enterotoxic genes in the end product? A long-term study for giardia and cryptosporium, including their possible seasonal variation, and development of methods for their detection in composted sludge, especially concentrating on their viability assessment, is needed.

6 CONCLUSIONS

1. *Salmonella*, *Listeria monocytogenes*, NLV-caliciviruses, astroviruses, *Giardia* and *Cryptosporidia* were all detected in the raw sludge samples. After mesophilic anaerobic digestion, after mechanical mixing with peat, and in 50% of the sludges after lime stabilisation they were still present. STEC and *Cambylobacter* were not found in the raw sludge samples.

During handling and application and after spreading in the field this kind of fertiliser is a potential hygienic risk to humans and domestic animals, especially if used in feed or vegetable production and if applied in a ground-water area or near watercourses.

Raw sewage sludge and poorly treated sludge products contain a wide variety of potential human and animal pathogens, and thus their use in agriculture is not recommended.

It is recommended that anaerobically digested sludge should be composted further in windrows or mattresses, with adequate turnings and/or aeration, or that thermal drying should be used to minimise its health risk

2. During mesophilic anaerobic digestion, inadequate lime stabilisation and poor composting the counts of sulphite-reducing clostridia, including *C. perfringens*, remained high or even increased in sludge products. Thus the products are not recommended as fertilisers on pig farms or pasture lands.

3. No *Salmonella*, *L. monocytogenes*, NLV caliciviruses or astroviruses were detected at 30 weeks in sewage sludge products after composting in open windrows. In two of the four composts, however, protozoal oocysts or cysts were still found.

Thus the recommended time for sludge composting in open windrows, with proper turnings and adequate addition of bulking agents, is at least six months.

4. In-vessel composting in drum or tunnel composting systems was not adequate to produce a pathogen-free sludge product. *L. monocytogenes* and protozoal cysts and oocysts and NLV caliciviruses were commonly detected in outfeed samples of these in-vessel composting plants, but no *Salmonella* were found.

After further composting of outfeeds in open windrows, caliciviruses and *L. monocytogenes* were not commonly found at the age of 10 weeks of the sludge, but protozoal cyst or oocysts were found even at 30 weeks.

Thus it is recommended that the end products of in-vessel composting plants should be further composted or cured in windrows or in mattresses, with adequate turnings, for at least six months.

5. Salmonella-free sludge products may commonly contain *L. monocytogenes*, NVL caliciviruses, astroviruses, *Giardia* and *Cryptosporidium*; thus *Salmonella* alone is not an adequate indicator organism for human pathogen risk monitoring in sludge products. At least the counts of *E. coli* and enterococci should also be monitored and the counts of them should be no higher than 100 and 1000 cfu/g_{dw}, respectively. However, according to our results, for instance, the outfeeds from drum composting plants may contain NLV caliciviruses and protozoal cysts even though *Salmonella*, *E. coli* or enterococci are not found.

Listeria monocytogenes and NVL caliciviruses were found to be also suitable for indicator organisms for checking the product for hygienic safety, as well as for evaluating the treatment process for its capability to inactivate pathogenic microbes which are of public health significance.

Giardia cysts and *Cryptosporidium* oocysts: more studies are needed on any seasonal variation in their occurrence in raw sludge. Methods in which larger sample volumes can be studied when the samples are solid and not suitable for IMS separation. Species determination and viability studies are important in risk characterisation and assessment.

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APPENDIX 1

Table 1. Wastewater treatment plants, sanitation method, composting method, process information and sampling.

Plant and type 1)	Sanitation method	Sanitation time, pH, temperature	Composting method	Composting information (bulking agents, turnings)	Samples (sludge "age")
1	Anaerobic digestion	20-21 days, 36-37°C	Mattress	Peat:bark:sludge (1:1:2 ;v/v/v) Machinery turnings	Raw sludge (0-3 days) Digested sludge (21 days) Composted (10 weeks) Composted (30 weeks)
2	Anaerobic digestion	20 days; 37.5 °C	Mattress	No bulking agent used, not turned	Raw sludge (0-3 days) Digested sludge (30 days) Composted (10 weeks) Composted (30 weeks)
3	Anaerobic digestion	21 days 34-36 °C	Windrow	Not turned	Raw sludge (0-3 days) Digested sludge (28 days) Composted (10 weeks) Composted (30 weeks)
4	Anaerobic digestion	20 days	Windrow Thermal drying	Turned 5 times (every third week)	Raw sludge (0-3 days) Digested sludge (28 days) Composted (10 weeks) Composted (30weeks) Dried (30days)
5	Lime	pH 11 30 min -> dewatering	-	-	Raw sludge (0-2 days) Limed sludge (3 days)
6	Lime	Dewatering - >pH 12; 1-2 h	-	-	Raw sludge (0-3 days) Limed sludge (4 days)
7	Lime	Dewatering -> pH 12-13; 30 min	-	-	Raw sludge (0-3 days) Limed sludge (4 days)
8	Lime	pH 11; 30 min-> dewatering	-	-	Raw sludge (0-3 days) Limed sludge (3 days)
9	No		No	Peat and sludge (1:1; v/v)	Raw sludge (0-3 days) Peat-sludge mix (3 days)
10	No		No	Peat, lime and sludge	Raw sludge (0-3 days) Peat-sludge mix (3 days)
13	No	-	Windrow	Bark and sludge	Raw sludge (0-3 days) Composted (10 weeks) Composted (30 weeks)
14	No	-	Windrow	Bark, peat, onion husk and sludge Turnings: after 2 weeks, after 1 month after two months	Raw sludge (0-3 days) Composted (10 weeks) Composted (30 weeks)
15	No		Windrow	Bark, wood chips and sludge (1:1:2;v/v/v) Bulking agents mixed after 3 months aging	Raw sludge (0-3 days) Composted (10 weeks) Composted (30 weeks)
16	No		Windrow	Sludge: bark (2:1) Turned after 3 months	Raw sludge (0-2 days) Composted (10 weeks) Composted (30 weeks)

17	Tunnel composting (2/6/19)	6 days	After tunnel in windrows	Coarse bark; Not turned	Raw sludge (0-3 days) Sanit. Compost (8 days) Composted (10 weeks) Composted (30 weeks)
18	Tunnel composting	8 + 6 days	After tunnel sieved and composted in windrows	Peat:wood chips:re-cycled chips:sludge (11:11:7:92;w/w) turned after 6 weeks	Raw sludge (0-3 days) Sanit. Compost (22 days) Composted (10 weeks) Composted (30 weeks)
19	Tunnel composting	16 days, not turned	After tunnel in windrows	Wood chips:sludge (1:1 v/v) Turned and sieved before the last sampling	Raw sludge (0-3 days) Stabil. compost (21days) Composted (10 weeks) Composted (30 weeks)
20	Tunnel composting (2/3/12)	4 + 11 days	After tunnel in windrows	Wood chips:sludge (2.3:1.5 v/v) Windrow not turned	Raw sludge (0-3 days) Sanit. compost (17days) Composted (10 weeks) Composted (30 weeks)
21	Drum ($100m^3$) composting	5 days	After drum in windrows	Peat:sludge (1.5:1 v/v)	Raw sludge (0-3 days) Sanit. compost (7 days) Composted (10 weeks) Composted (30 weeks)
22	Drum composting	4 days	After drum in windrows	Cutter chips: peat: sludge (1.5:0.5:1) Turned twice	Raw sludge (0-3 days) Sanit. compost (7 days) Composted (10 weeks) Composted (30weeks)
23	Drum ($125m^3$) composting	14 days	After drum in windrows	Peat:sludge (1.8:1)	Raw sludge (0-3 days) Sanit. compost(15 days) Composted (10 weeks) Composted (30 weeks)
24	Drum ($80m^3$) & tunnel composting	14 days	After drum and tunnel in windrows	Cutter chips:sludge (4:7 v/v) 1.5-2 m height	Raw sludge (0-3 days) Sanit. compost(16 days) Composted (10 weeks) Composted (30 weeks)

Table 2. *Chemical and physical characteristics for the sludge and sludge product samples*

Wastewater treatment plant and sample type	Sample Code	Temperature during sampling, °C	pH (1+5)	Dry matter %	LOI $\%_{dw}$	Conductivity (EN 13038) mS/m
1						
Raw sludge	1242		6,8*)	4	72	n.m.
Digested	1480		7,7*)	2	56	n.m.
Compost10	2159	52.4	7.8	39	67	68.3
Compost30	3883	49.4	8.7	32	44	119
2						
Raw sludge	1345		6.5*)	4	68	n.m.
Digested	1615		7.6*)	2	53	n. m.
Compost10	2318	10.2	8.0	30	51	133
Compost30	4031	11.3	4.6	34	40	153
3						
Raw sludge	1246		7.1*)	3	70	n.m.
Digested	1546		7.3*)	3	52	n.m.
Compost10	2160	8.8	7.2	31	40	124
Compost30	3957		6.6	39	44	92.4
4						
Raw sludge	1248		6.5*)	3	71	n.m.
Digested	1547		7.6*)	2	55	n.m.
Compost10	2131		8.2	33	56	226
Compost30	3958	27.1	5.7	31	48	101
Dried thermally	2133		7.1	91	57	80.8
5						
Raw sludge	2058		7.2*)	<1	63	n.m.
Limed	2059		8.5*)	5	55	n.m.
6						
Raw sludge	1129		6.7*)	3	70	n.m.
Limed	1130		12.3	26	47	278
7						
Raw sludge	1131		6.7*)	5	75	n.m.
Limed	1132		11.1	19	66	55.7
8						
Raw sludge	2060		7.3	5	65	n.m.
Limed	2061		7.7	5	65	n.m.
9						
Raw sludge	1101		7.2	9	61	n.m.
Peat mixture	1836		8.1	26	64	72.1
10						
Raw sludge	1096		6.6	2	71	n.m.
Peat mixture	1167		7.0	27	57	77.7
13						
Raw sludge	1100		7.6	3	66	n.m.
Compost10	2040	77.1	7.3	39	78	52.6
Compost30	3776	46.1	5.6	27	74	33.5
14						
Raw sludge	1372		6.9	3	75	n.m.
Compost10	2303	42.3	7.7	38	75	116
Compost30	4091	50.2	7.0	28	62	28.5

15						
Raw sludge	1099		6.3	4	71	n.m.
Compost10	2078	8.4	7.5	19	72	214
Compost30	3777	26.9	5.7	23	75	37.7
16						
Raw sludge	1245		6.9	12	68	n.m.
Compost10	2130		6.9	25	81	143
Compost30	3956	33.1	4.9	28	74	28.4
17						
Raw sludge	1369		7.1	3	69	n.m.
Outfeed	1425	54	6.1	29	84	34.7
Compost10	2255	31.3	7.2	42	81	33.6
Compost30	n.s					
18						
Raw sludge	1363		6.5	6	77	n.m.
Outfeed	1564	40	7.6	55	76	127
Compost10	2305	59	7.4	83	74	147
Compost30	4109	48	6.9	44	64	142
19						
Raw sludge	1373		6.9	5	67	n.m.
Outfeed	1565	20-56	7.7	53	65	243
Compost10	2302	27.5	7.8	82	59	113
Compost30	4093	17.3	6.6	49	49	151
20						
Raw sludge	1374		7.4	3	65	n.m.
Outfeed	1515		7.8	39	80	41.2
Compost10	2304	54.3	7.4	42	78	86.5
Compost30	4092	22.7	6.6	41	73	15.9
21						
Raw sludge	1097		7.8	3	60	n.m.
Outfeed	1168		7.5	27	84	58.3
Compost10	2052		6.6	27	82	57.8
Compost30	3806	19.3	4.8	25	78	47.5
22						
Raw sludge	1247		6.3	2	81	n.m.
Outfeed	1295	45	7.5	25	94	96.2
Compost10	2129	34.1	7.5	27	93	78.5
Compost30	3959	30.9	6.3	31	89	94.6
23						
Raw sludge	1098		7.1	3	63	n.m.
Outfeed	1296	47	7.6	32	86	45.4
Compost10	2053		7.6	29	84	36.1
Compost30	3805	39.7	7.1	27	79	50.6
24						
Raw sludge	1371		6.6	6	72	n.m.
Outfeed	1500		8.1	46	86	270
Compost10	2256	55	7.5	43	86	73.7
Compost30	4010	39-53	5.0	36	81	102

*) pH measured without dilution

Table 3. *Levels of Clostridium perfringens, sulphite-reducing clostridia, enterococci and E. coli (log cfu/gdw) and Salmonella bacteria (serotype in parenthesis) found in sewage sludge and sludge product samples from 22 wastewater treatment plants.*

Wastewater treatment plant	Code	*Clostridium perfingens*	Clostridia	Enterococci	*E.coli*	*Salmonella* found in 25g (serotype)
1						
Raw sludge	1242	<2.5	5.38	5.85	6.24	+ (*S.* Agona, *S.* Newport, *S.* Mbandaka)
Digested	1480	5.15	6.44	3.48	<3.0	+ (*S.* Infantis)
Compost10	2159	<1.5	<4.0	1.89	<1.5	-
Compost30	3883	4.29	4.99	3.00	1.67	-
2						
Raw sludge	1345	4.80	6.10	5.32	5.27	+ (*S.* Brandenburg, *S.* Hadar)
Digested	1615	5.57	6.74	3.88	3.93	+ (*S.*Haifa)
Compost10	2318	5.64	5.75	2.87	<1.5	-
Compost30	4031	5.51	5.64	<1.5	1.77	-
3						
Raw sludge	1246	<2.5	5.82	4.67	5.44	+ (*S.* Agona, *S.* Ealing, *S.* Javiana, S. Newport)
Digested	1546	6.40	6.56	3.00	<2.5	+ (*S.* Enteritidis)
Compost10	2160	5.62	5.76	2.29	2.87	-
Compost30	3957	2.89	3.26	<1.5	2.01	-
4						
Raw sludge	1248	5.54	5.75	4.22	4.04	+ (*S.* Sandiego, *S.* Derby)
Digested	1547	6.11	6.33	3.40	3.10	+ (*S.* Sandiego, *S.* Tenesseee)
Compost10	2131	5.71	5.93	3.60	<1.5	-
Compost30	3958	5.55	5.71	<1.5	1.51	-
Dried thermally	2133	<1	3.51	<1.0	<1.0	-
5						
Raw sludge	2058	6.15	6.30	5.34	6.57	+ (*S.* Virchow)
Limed	2059	6.34	6.38	5.93	5.27	+ (*S.* Virchow)
6						
Raw sludge	1129	5.87	6.01	5.56	5.60	+ (*S.* Ebrie, *S.* Agona)
Limed	1130	<3.0	3.02	<2.0	<2.0	-
7						
Raw sludge	1131	<2.5	6.78	5.45	4.75	+ (*S.* Infantis)
Limed	1132	3.33	3.84	<3.5	<2.0	-
8						
Raw sludge	2060	6.45	6.48	5.34	5.87	+ (*S.* Senftenberg)
Limed	2061	6.26	6.41	5.58	6.00	+ (*S.* Senftenberg)
9						
Raw sludge	1101	6.12	6.46	6.02	5.99	+ (*S.* Enteritidis RDNC)
Peat mixture	1836	5.36	5.49	3.84	2.76	-
10						
Raw sludge	1096	<3.0	5.37	4.74	4.74	+ (*S.* Coeln, *S.* Senftenberg)
Peat mixture	1167	<2.0	4.47	4.68	3.80	+ (*S.* Senftenberg)

13						
Raw sludge	1100	<2.5	5.88	5.09	5.95	+ (*S*. Newport, *S*. Senftenberg)
Compost10	2040	4.31	4.49	3,98	<1.5	-
Compost30	3776	5.12	5.12	2.61	<2.0	-
14						
Raw sludge	1372	6.14	6.52	5.38	5.60	+ (*S*. Enteritidis PT RDNC)
Compost10	2303	<1.5	<1.5	5.41	3.25	-
Compost30	4091	3.34	3.46	<2.0	<2.0	-
15						
Raw sludge	1099	<2.5	5.78	4.51	5.00	+ (*S*. Anatum)
Compost10	2078	5.98	5.98	3.47	2.37	-
Compost30	3777	2.98	3.28	<2.0	<2.0	-
16						
Raw sludge	1245	<2.0	5.65	4.90	4.77	+ (*S*. ssp I)
Compost10	2130	<2.0	5.60	5.42	2.98	+ (*S*. Infantis)
Compost30	3956	2.93	3.23	3.49	<2.0	-
17						
Raw sludge	1369	5.67	5.82	4.94	4.56	+ (*S*.Typhimurium)
Outfeed	1425	4.58	4.79	3.82	3.50	-
Compost10	2255	4.08	4.68	4.22	5.44	-
Compost30	n.s.					
18						
Raw sludge	1363	5.97	6.23	6.40	5.85	+ (*S*. Poona, *S*. Virchow)
Outfeed	1564	<1.5	2.76	5.37	3.30	-
Compost10	2305	<1.0	1.08	<1.0	<1.0	-
Compost30	4109	<1.5	<1.5	1.98	<1.5	-
19						
Raw sludge	1373	6.15	6.34	6.06	5.92	+ (*S*. Anatum)
Outfeed	1565	<1.5	<1.5	3.42	1.58	-
Compost10	2302	<1.0	<1.0	3.89	3.75	-
Compost30	4093	<1.5	<1.5	4.31	3.12	-
20						
Raw sludge	1374	<2.5	6.43	4.80	5.01	-
Outfeed	1515	<1.5	<1.5	6.27	5.38	-
Compost10	2304	<1.5	5.22	5.31	4.23	-
Compost30	4092	<1.5		2.09	1.79	-
21						
Raw sludge	1097	<2.5	6.92	5.94	5.48	+ (*S*. Newport, *S*. Typhimurium DT 104B)
Outfeed	1168	4.82	5.18	1.57	<2.0	-
Compost10	2052	4.71	4.89	2.71	<2.0	-
Compost30	3806	4.03	4.39	<2.0	<2.0	-
22						
Raw sludge	1247	2.70	6.32	4.81	5.31	+ (*S*. Newport)
Outfeed	1295	<2.0	<2.0	4.33	2.48	-
Compost10	2129	<2.0	2.35	<2.0	<2.0	-
Compost30	3959	<2.0	3.79	<2.0	<2.0	-
23						
Raw sludge	1098	<2.5	6.34	4.43	5.67	+ (*S*. Typhimurium DT 104)
Outfeed	1296	4.27	4.61	5.28	<1.5	-
Compost10	2053	4.82	5.06	3.37	<1.5	-
Compost30	3805	<2.0	4.77	<2.0	<2.0	-
24						
Raw sludge	1371	6.77	7.07	5.03	3.99	+ (*S*. Branderup, *S*. Hadar)
Outfeed	1500	<1.5	<1.5	<1.5	<1.5	-
Compost10	2256	<1.5	<1.5	<1.5	<1.5	-
Compost30	4010	<1.5	<1.5	<1.5	<1.5	-

Table 4. *Salmonella isolated from the sludge and sludge product samples: serotypes and antimicrobial susceptibility*

Wastewater treatment plant	Code	Salmonella serotype and phage type	Resistance type
1			
Raw sludge	1242	*S*. Agona	
		S. Mbandaka	Nx
		S. Newport	Nx
Digested	1480	*S*. Infantis	
2			
Raw sludge	1345	*S*. Hadar	NxT
		S. Brandenburg	
Digested	1615	*S*. Haifa	NxSxtSuTTm
3			
Raw sludge	1246	*S*. Agona	
		S. Ealing	
		S. Javiana	
		S. Newport	Nx
Digested	1546	*S*. Enteritidis PT1	
4			
Raw sludge	1248	*S*. Sandiego	
		S. Derby	
Digested	1547	*S*. Sandiego	
		S. Tenesseee	
5			
Raw sludge	2058	*S*. Virchow	
Limed	2059	*S*. Virchow	
6			
Raw sludge	1129	*S*. Ebrie	
		S. Agona	Nx
7			
Raw sludge	1131	*S*. Infantis	
8			
Raw sludge	2060	*S*. Senftenberg	SxtSuTm
Limed	2061	*S*. Senftenberg	SxtSuTm
9			
Raw sludge	1101	*S*. Enteritidis PT8	
10			
Raw sludge	1096	*S*. Coeln	
		S. Senftenberg	SxtSuTm
Peat mixture	1167	*S*. Senftenberg	SxtSuTm
13			
Raw sludge	1100	*S*. Senftenberg	n.d.
		S. Newport	
14			
Raw sludge	1372	*S*. Enteritidis PT RDNC	
15			
Raw sludge	1099	*S*. Anatum	Nx
16			
Raw sludge	1245	*S*. ssp I	
Compost10	2130	*S*. Infantis	
17			
Raw sludge	1369	*S*. Typhimurium DT40	
18			
Raw sludge	1363	*S*. Poona	
		S. Virchow	(Su)

Waste water treatment plant	Code	Salmonella serotype and phage type	Resistance type
19 Raw sludge	1373	*S.* Anatum	
21 Raw sludge	1097	*S.* Typhimurium DT104B *S.* Newport	CSuSTA
22 Raw sludge	1247	*S.* Newport	
23 Raw sludge	1098	*S.* Typhimurium DT104	CSuSTA
24 Raw sludge	1371	*S.* Branderup *S.* Hadar	SxtSuSTTm NxTA

n.d. not done

Antibiotics:

A	Ambicillin
G	Gentamicin
Ctx	Ceftotaxime
C	Chloramphenicol
Nx	Nalidixic acid
Cp	Ciprofloxacin
Sxt	Sulphonamides + Trimetoprim
Su	Sulphonamides
S	Streptomycin
T	Tetracycline
Tm	Trimetoprim

Table 5. *Pathogenic bacteria, viruses and protozoa found in the sludge and sludge product samples from 22 wastewater treatment plants.*

Plant number and sample types	Code	*Crypto-sprodium parvum* 1 g	*Giardia* 1 g	*Mykobacter* sp. 4 g	NVL-calicivirus Genogroup I 20 - 25 g	NVL-calicivirus Genogroup II 20 - 25 g	Astrovirus 20 - 25 g	*Listeria monocyto-genes* 25 g
1								
Raw sludge	1242	3+/3	2+/3		+[1)] (1:10)	+[1)] (1:10)		2+/3
Digested	1480	+	-		-	+ (1:10)		-
Compost10	2159	-	+	+	-	-		-
Compost30	3883	-	+		-	-		-
2								
Raw sludge	1345	2+/3	2+/3		+[1)] (1:10)	+[1)] (>1:1000)		3+/3
Digested	1615	+	-		+ (>1:10)	+ (>1:1000)	+ (1:100)	+
Compost10	2318	-	+	*M.terrae*		+ (>1:100)	+ (≥1:100)	-
Compost30	4031	-	+			-	-	-
3								
Raw sludge	1246	0+/3	0+/3		+[1)] (1:10)	+[1)] (1:10)	+ [1)] (1:1000)	3+/3
Digested	1546	-	-		-	+ (1:1000)	+ (1:10)	-
Compost10	2160	-	+	+		-	+ (≥1:100)	-
Compost30	3967	-	+			-	-	-
4								
Raw sludge	1248	2+/3	0+/3		+[1)] (1:10)	+[1)] (1:10)		3+/3
Digested	1547	-	-		+ (1:1)	+ (1:100)		-
Compost10	2131	-	+	*M.terrae*		-		-
Compost30	3958	-	+			-		-
Dried thermally	2133	-	-	-		-		-
5								
Raw sludge	2058	1+/2(2+/2	0+/2(1+/2		-[1)]	+[1)] (1:100)	-[1)]	1+/2
Limed	2059	0+/1(-)	1+/1	*M.terrae*		+ (1:10)	+(≥1:1000)	(1)/+
6								
Raw sludge	1129	1+/1	0+/		+[1)] (1:10)	-[1)]		3+/3
Limed	1130	-	-	+		-		-
7								
Raw sludge	1131	1+/3	1+/3		+[1)] (1:10)	+[1)] (1:100)		2+/3
Limed	1132	-	-	-	-	-		(1)/+
8								
Raw sludge	2060	0+/3	2+/3		+[1)] (1:1)	+[1)] (>1:1000)	+[1)] (≥1:1000)	3+/3
Limed	2061	(3+/3) 0+/1	1+/1	-	+ (>1:10)	+ (>1:1000)	+(≥1:1000)	+
9								
Raw sludge	1101	3+/3	0+/3		+[1)] (1:10)	+[1)] (>1:100)		3+/3
Peat mixture	1836	+	+	*M.terrae*		+ (>1:100)		+
10								
Raw sludge	1096	1+/3	2+/3		+[1)] (1:1)	+[1)] (1:10)		3+/3
Peat mixture	1167	-	+	+		+ (1:1)		-
13								
Raw sludge	1100	1+/3	2+/3		+[1)] (1:1)	+[1)] (1:10)		3+/3
Compost10	2040	-	-	+		-		-
Compost30	3776	-	-			-		-
14								
Raw sludge	1372	1+/3	1+/3		+[1)] (1:1)	+[1)] (1:10)		3+/3
Compost10	2303	+	+	+		-		+
Compost30	4091	-	-			-		-

15								
Raw sludge	1099	1+/3	1+/3		-[1)]	-[1)]	+[1)] (1:10)	3+/3+
Compost10	2078	-(0+/1PCR)	-	*M.terrae*		+ (>1:100)	+ (≥1:100)	-
Compost30	3777	+	-			-	-	
16								
Raw sludge	1245	0+/2	0+/2		+[1)] (1:10)	+[1)] (1:100)	+[1)] (1:1000)	3+/2
Compost10	2130	+	+	+		+ (1:10)	+ (1:100)	+
Compost30	3956	-	+			-	-	-
17								
Raw sludge	1369	3+/3	2+/3		+[1)] (1:1)	+[1)] (1:10)	+[1)] (≥1:100)	3+/3
Outfeed	1425			nd		+ (1:10)	+ (≥1:100)	+
Compost10	2255	-	-	*M.terrae*		-	+ (≥1:100)	+
Compost30	no							
18								
Raw sludge	1363	2+/3	1+/3		+[1)] (1:1)	+[1)] (1:100)	+[1)] (1:1000)	3+/3
Outfeed	1564	-	-	+		-	-	-
Compost10	2305	-	-	+		-	-	-
Compost30	4109	-	-			-		-
19								
Raw sludge	1373	2+/3	2+/3		+[1)] (1:1)	+[1)] (1:10)		3+/3
Outfeed	1565	-	-	+		-		+
Compost10	2302	+	-	+		-		+
Compost30	4093	-	-			-		-
20								
Raw sludge	1374	3+/3	1+/3		+[1)] (1:1)	+[1)] (1:1)		3+/3
Outfeed	1515	-	+	+		-		-
Compost10	2304	-	-	*M.terrae*		-		-
Compost30	4092	-	-			-		-
21								
Raw sludge	1097	1+/3	0+/3		+[1)] (1:1)	+[1)] (1:10)		3+/3
Outfeed	1168	+	+	+		+ (1:10)		-
Compost10	2052	+	-	*M.terrae*		-		-
Compost30	3806					-		-
22								
Raw sludge	1247	1+/3	0+/3		-[1)]	+[1)] (1:1)		3+/3
Outfeed	1295			+		+ (1:1)		-
Compost10	2129	+-	+	*M.terrae*		-		-
Compost30	3959	-	+			-		-
23								
Raw sludge	1098	3+/3	1+/3		+[1)] (1:1)	+[1)] (1:10)	+[1)] (1:1)	3+/3
Outfeed	1296			-		+ (1:10)		-
Compost10	2053	-	-	+		-	-	-
Compost30	3805	-	-			-	-	-
24								
Raw sludge	1371	3+/3	0+/3		+[1)] (1:10)	+[1)] (1:10)	+[1)] (≥1:100)	3+/3
Outfeed	1500	-	+	+		+ (1:10)	-	-
Compost10	2256	+	+	+		-	-	-
Compost30	4010	-	-			-	-	-

[1)] Only one of the three successive sludge samples was analysed

APPENDIX 2 Detailed descriptions of the wastewater and sludge treatment plants

Plant 1

In 2001, 97,600,000 m^3 of waste water was treated on the plant with the activated sludge process including mechanical, biological and chemical P and N removal. Mixed raw sewage sludge (mostly raw primary sludge and excess sludge, and a little activated sludge) was digested for 20-21 days at 36-37°C. In 2001, 54,000 tons of dewatered digested sludge (32% DM) was composted in an open-air 2 m high and 10 wide mattress, peat and bark as bulking agents in a ratio 2:1:1. During the experiment, mattress was machinery turned once in a month. Temperatures were not measured during composting.

The average temperature measured at the first sampling was 52.4°C (n=4) and at the final sampling 48.3°C (n=9).

Plant 2

In 2001, 3,900,000 m^3 of waste water was treated on the plant with the simultaneous biological and chemical P and N removal. Mixed raw sewage sludge (mostly raw primary sludge and excess sludge; 5%DM) was digested for 28 days at 37°C. In 2001, 3,200 m^3 of dewatered digested sludge (30%DM) was heaped to a flat mattress-like open-air windrow (height 1 m, width 10 m) without bulking agent addition and without turnings during the composting process.

The temperature measured at the first sampling was 10.2°C and at the final sampling 11.3°C.

Plant 3

In 2001, 8,400,000 m^3 of waste water was treated on the plant with the simultaneous biological and chemical precipitation with total N removal. The total amount of 111,100 m^3 of mixed raw sewage sludge (raw primary sludge and excess sludge in a 1:1 ratio; 4-5% DM) was digested for 28 days at 34-36°C. In 2001, 16,400 m^3 of dewatered digested sludge was composted in open-air windrows. The windrows were not turned, and temperatures were not measured during the composting.

The temperature measured at the first sampling was 8.8°C and at the final sampling 18.1°C (n=4).

Plant 4

In 2001, about 4,700,000 m^3 of waste water was treated on the plant with the simultaneous biological and chemical P and N removal. Excess sludge (5%DM) was digested for 28 days at 35-37°C. In 2001, about 7,000-8,000 tons of dewatered digested sludge (20-25%DM) was divided in two parts; the one was composted in open-air windrows, with wood chips as a bulking agent, and the rest of the sludge was dried further in a thermal drying system for 40-80 min in air flow with the air temperature at 120-140°C to approximately a dry weight of 85%. The windrow was turned five times during the experiment (every third week). Temperatures were not measured during the composting.

The temperature, in the experimental windrow, was at the final sampling 27.1°C (n=4).

Plant 5

In 2001, about 377,000 m^3 of waste water was treated on the plant with the biological and chemical P removal. Mixed raw sludge is treated during thickening with quicklime to rise the sludge pH to 11; after a few days the limed sludge was dewatered (17%DM) and used in agriculture within a year.

Plant 6

Waste water was treated at the plant with the simultaneous biological and chemical P and N removal. After dewatering to 17-18%DM, quick lime was added to rise the sludge pH 12 for 1-2

h with continuous mixing. Lime stabilised sludge was heaped in the field, stored from the spring to the autumn, and then used in agriculture.

Plant 7

Waste water was treated onthe plant with the simultaneous biological and chemical P and N removal. During dewatering to 17-18%DM, quick lime was mixed with the sludge to rise the pH 12 for 30 minutes. The lime stabilised sludge was used next day in agriculture.

Plant 8

The waste water was treated on the plant with the simultaneous biological and chemical precipitation and N removal with denitrfication. Mixed raw sludge (a mixture of active and excess sludge) is treated with quicklime to rise the pH to 11, and dewatered (16%DM) after 30 minutes. The plant has a catchment area of 11 000 inhabitants.

Plant 9

In 2001, 960,000 m^3 waste water was treated on the plant with the simultaneous biological and chemical precipitation (a mixture of active and excess sludge). Mixed raw sludge is treated with lime and dewatered (22%DM). In 2001, 2,400 m^3 dewatered sludge was mixed with *Sphagnum* peat in a ratio 1:1 (v/v), and used in agriculture without composting.

Plant 10

The waste water was treated on the plant with the simultaneous biological and chemical P removal. Mixed raw sludge is treated with lime and dewatered. In 2001, about 14,000 m^3 dewatered sludge was mixed with *Sphagnum* peat in a ratio 1:1 (v/v), and used in agriculture without composting.

Plants 11 and 12 not found to the experiment

Plant 13

In 2001, 4,400,000 m^3 of waste water was treated on the plant with the simultaneous biological P and N removal. About 6800 m^3 dewatered mixture of active and excess sludge with an average dry matter content of 15% was composted in open-air windrows with bark as a bulking agent (sludge-bark ratio 1:2). The windrows were 2-2.5 m high, 6 m wide and 100 m long. They were turned first after the three weeks of composting and after that, once in a month.

The temperature measured at the first sampling (at the age of 10 weeks) was 77.1°C, 73 °C at the age of 23 weeks, and at the final sampling 46.1°C (n=4).

Plant 14

On the plant waste water was treated with the biological N removal (about 60-70% of N lost) and chemical P removal. Lime is not used. In 2001, about 1500 m^3 of the dewatered excess sludge (17-18%DM) was composted in open-air windrows with bark, peat and onion husk as bulking agents. The windrows were 2.5-3 m high and 4 m wide, they were turned with an"allu"-machine at the age of two weeks, and after that turned after one months', three months' and five months' curing. The compost was used at the age of 6 months in green gardening.

The temperature measured at the first sampling was 42.3°C and at the final sampling 50.2°C (n=4).

Plant 15

The waste water was treated on the plant with the simultaneous biological and chemical P removal. Mixed sludge (raw sludge and two different kinds of excess sludge) was dewatered (16%DM) without lime addition, and composted in open-air windrows with bark and wood chips as bulking agents in a ratio of 2:1:1. During this study, the dewatered sludge was mixed with bulking agents just after the first sampling and no turnings were done since that.

The temperature measured at the first sampling was 8.4°C and at the final sampling 26.9°C (n=4).

Plant 16

In 2001, about 4,700,000 m^3 of waste water was treated on the plant with the simultaneous biological and chemical P and N removal. After dewatering, 7,200 m^3 of the raw sludge mixture (18%DM) was composted in open-air windrows with bark as a bulking agent (2:1). The windows were 2 –2.5 m high and 3 m wide, and they were turned three times per year, the first turn at the age of 3-5 months.

The temperature in the windrow was 33.1°C during the last sampling.

Plant 17

In 2001, about 870,000 m^3 of waste water was treated on the plant with the simultaneous biological and chemical P removal. About 9700 m^3 of raw sludge (a mixture of biosludge and chemical sludge; 1.6%DM) was dewatered to an average of 15%DM) and mixed with coarse bark chips (1:2) with a front loader and fed to a 6 m long and 2.5 m wide tunnel to a height of 2.5 m and composted six days. The tunnel was constantly force-aerated from the bottom throughout the length of the tunnel. After one days aeration, the feed back system kept the mean temperature in the compost at 55°C. At the day of the outfeeding the mean temperature in the tunnel was 54°C. During sanitation the material was not turned.

After the sanitation phase of 6 days, the compost was moved to the composting field for further composting in open windrows. During windrow composting the mass was not turned. Before the last sampling the experimental windrow was disappeared from the composting field.

The temperature in the windrow was 31.3°C during the sampling of the 10 weeks old compost.

Plant 18

In 2001, about 6,500,000 m^3 of waste water was treated on the plant with the simultaneous biological and chemical P and N removal. Annually, 20,000 m^3 of the raw sludge(4-6%DM) was dewatered by a centrifuge to 25%DM. In 2001, 8 100 m^3 of the sludge was composted with wood chips and peat as bulking agents in a ratio of 1:

Table 1. *The temperatures measured during curing at plant 18.*

Date	Temperature °C
10.5.2001	65
14.5.2001	58
21.5.2001	69
13.6.2001	59
16.10.2001	48

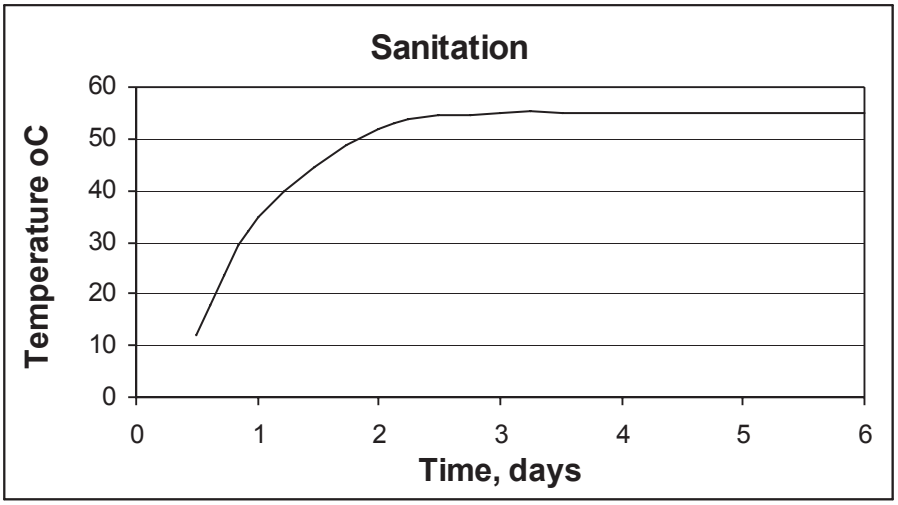

Figure 1. *Mean temperature (n=3) as a function of time during sanitation process measured at the tunnel composting plant 17.*

Figure 2. Mean temperature (n=3) as a function of time during sanitation process with two phases, measured at the tunnels 1 and 2.

0.8:0 in a tunnel composting system with two tunnels (6 m high, 6m wide and 20 m long). In this study, the total mass of the infeed was 121 tons (92 tons sludge, 11 tons peat, 11 tons chips and 7 tons recycled chips). After a total of 8 days in the tunnel 1, the compost (then 63 tons) was moved to the tunnel 2 in order to mix the material. After the total sanitation phase of 15 days, the compost (58 tons) was outfed and sieved (20 mm) and heaped (24.4.01) for further composting in an open windrow (2.5 m high, 4-5 m wide). The windrow was twice turned during this study.

Plant 19

In 2001, about 3,600,000 m^3 of waste water was treated on the plant with the simultaneous biological and chemical P and N removal. In 2001, about 4300 m^3 of dewatered sludge was composted with wood chips in a ratio of 1:1 in a tunnel composting system (with 3 tunnels). About 200-250 m^3 of the mass was fed per tunnel and was not turned during the processing. After the total sanitation phase of 21 days, the compost 16 days, the compost was moved to the composting field, sieved (20 mm) and

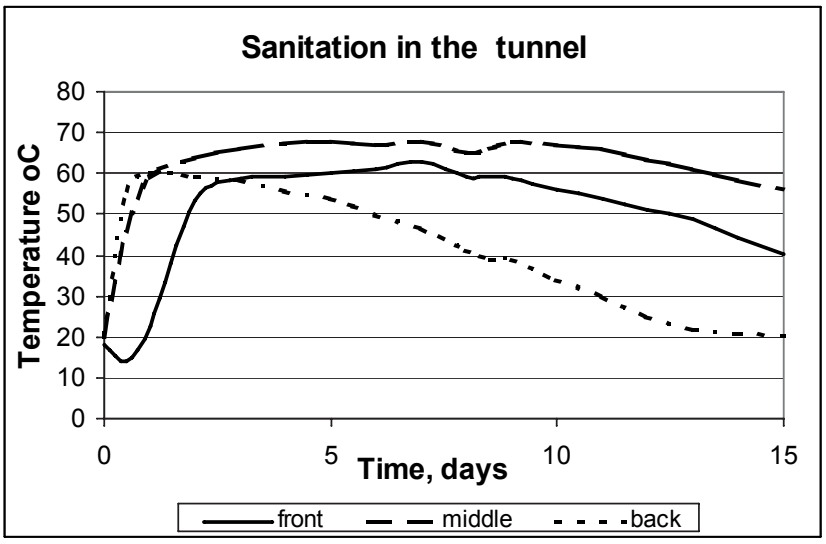

Figure 3. Temperature as a function of time during sanitation process measured in three different points at the tunnel composting plant 19.

heaped for further composting in open-air windrows (2.5 m high and 4-5 m wide). The windrow was turned twice during the experiment.

Plant 20

In 2001, about 48,800 m^3 of waste water was treated on the plant with the simultaneous biological and chemical P and N removal. After dewatering, the raw sludge mixture (about 18-20%DM) was mixed with deciduous wood chips (15 m^3 + 23 m^3) and heaped into a tunnel composting system with three tunnels (each tunnel 5.8 m high, 6 m wide and 20 m long). After a total of 4 days in the tunnel 1, the experimental compost was moved to the tunnel 2 in order to mix the material. After a total sanitation phase of 15 days, the compost was moved to the composting field and heaped for curing in an open-air windrow (2.5 high and 4-5 m wide). The windrow was not turned during the experiment, but it was sieved (20 mm) after the final sampling at the age of 30 weeks. Temperatures were not measured during the windrow composting.

In 2001, about 600 m^3 dewatered sewage sludge, 1100 m^3 catering biowaste and 2000 m^3 wood chips (including 40% re-circulated chips) were processed in the composting plant.

Plant 21

In 2001, about 1,200,000 m^3 of waste water was treated on the plant with the simultaneous biological and chemical P removal. After dewatering, the raw sludge mixture (about 18%DM) was mixed with peat (1:1.5) and continuously fed into a drum composting system (a total volume of 100 m^3). No temperature recording was done during the process, but manually measured now and then (mean about 40°C). After the total sanitation phase of 7 days in the drum, the outfeed was moved to the composting field and heaped for further curing in an open-air windrow. During the experiment, the windrow was not turned and temperature not measured.

Plant 22

In 2001, about 500,000 m^3 of waste water was treated at the plant with the simultaneous biological and chemical P and N removal. Annually, about 2300 m^3 of raw sludge mixture was dewatered to11%DM and composted in a drum composting system (two 90 m^3 drums) with cutter chips and peat as bulking agents in a ratio 1:1.5:0.5. Temperature was now and then manually measured during the sanitation process; the mean being

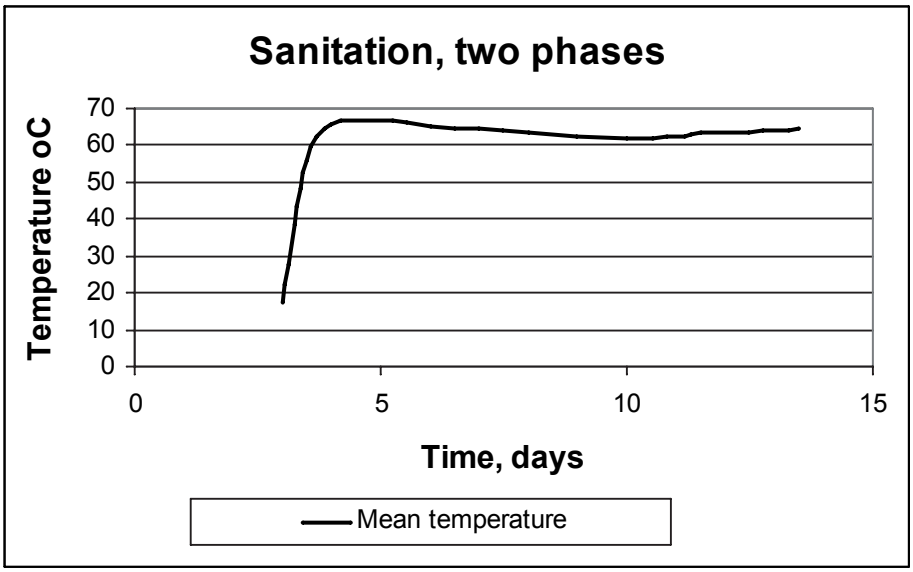

Figure 4. *Temperature as a function of time during sanitation process with two phases measured at the tunnel composting plant 20. No temperature data available from tunnel 1, phase 1.*

about 45-50°C in the middle of the drum during the experiment. After the total sanitation phase of 7 days, the outfeed was moved to the composting field and heaped for further curing in an open-air windrow. The windrow was turned 4 times during this experiment. Temperatures were not measured during the curing.

The temperature measured at the first sampling was 34.1°C and at the final sampling 30.9°C (n=4).

Plant 23

In 2001, about 10,700 m^3 of waste water was treated on the waste water treatment plant with the simultaneous biological P and N removal. Annually, about 1200 m^3 of dewatered sludge (23%DM) was mixed with light *Sphagnum* peat (1:1.8) and fed into a continuously working drum composting system (125 m^3). Temperature was measured once a week at three different points from the composting mass during the process in the drum. During the study, the temperature was 44.3°C in the middle and 47.8°C in the end of the drum. After the total sanitation phase of 15 days in the drum, the outfeed was daily heaped for further curing in an open-air windrow. The windrow was not turned, but temperatures were measured (the means were about 70 – 50 – 40°C beginning from the outfeed to the finished compost).

During the final sampling, at the age of 30 weeks, the temperature in the windrow was 39.7°C.

Plant 24

In 2001, about 2,300,000 m^3 of waste water was treated on the plant with the simultaneous biological and chemical P and N removal. Annually, after dewatering to 20%DM about 4000 m^3 of mixed raw sludge was composted in a drum composting system (two 80 m^3 drums) with fine cutter chips (7:4). The continuously working drum was fed during the three first days, and again during the 7th and 8th days. After the drum composting of 12 days, the outfeed was further composted a couple of days in a tunnel and heaped from the tunnel once a week for further curing into open-air windrows (1,5-2 m high and 2 m wide). During curing the windrows were not turned.

The temperature measured at the age of 10 weeks was 55.1°C and at the time of final sampling 46.1°C (n=4).

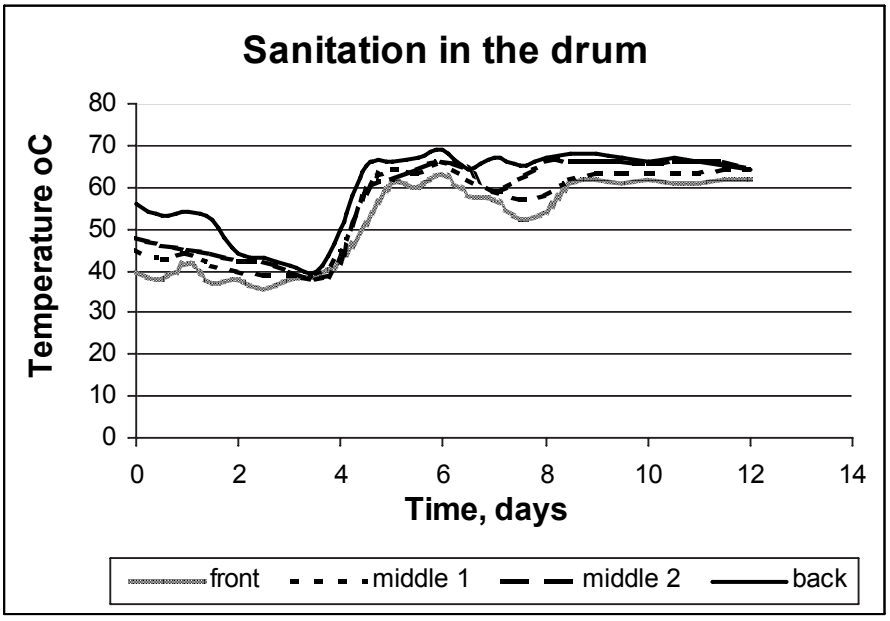

Figure 5. *Temperature as a function of time measured during sanitation process in the drum at the plant 24.*

APPENDIX 3 VALIDATION OF METHODS

3.1 Validation of detection of STEC by PCR in sewage sludge and sludge products

Marjut Eklund and Anja Siitonen, National Public Health Institute (KTL), Laboratory of Enteric Pathogens (LEP)

Procedure

The combination of the culture on SMAC and culture in mTSB and PCR for detection of STEC was validated by spiking two sewage samples with known colony forming units (cfus) of stx_1 and stx_2 positive STEC O157:H7 strain (ATCC 43895). Of the two sewage samples selected for spiking, one represented scanty (01/4010) and the other abundant (01/4109) original microbial growth on SMAC plates. First, serial dilutions (10^{-1} to 10^{-10}) of an overnight growth of the *stx* positive control strain in mTSB was made, and 100 µl of undiluted growth and of each dilution were cultivated on SMAC plates for determining cfu/ml. Simultaneously, 100 µl was similarly also inoculated into 10 bottles containing 10 g of the sewage giving scanty or abundant microbial growth on SMAC (in total 20 bottles). The spiked samples were shaken and cultivated on SMAC plates with a 10 µl loop. After this, 90 ml of mTSB was added into each bottle. The agar plates and broth bottles were grown at +37°C overnight. Cfu/ml of the *stx* positive control strain was calculated. The growth on all SMAC plates and in mTSB bottles of the spiked cultures were studied for the stx_1 and stx_2 genes of STEC by PCR as described above. In addition, latex agglutination of the cultures was executed with the *E. coli* O157 antigen kit (Oxoid, Hampshire, England).

RESULTS

Spiked sewage samples

The number of the viable stx_1 and stx_2 positive control bacteria after overnight incubation in mTSB was about 10^{10} cfu/ml, resulting in about 10^8 cfu/g to <1 cfu/g in the spiked sewage samples.

In the sewage with original scanty growth on SMAC plates, the detection limit of PCR for stx_1 and stx_2 was 10^4 cfu/g, corresponding to 100 cfu on the SMAC plate. In addition, the growth on one SMAC plate cultivated from the dilution that corresponded to 10 cfu/g of sewage and less than one colony on SMAC was *stx* positive in PCR. The latex O157 test was done from the same growth areas as PCR. All the samples which were positive in PCR agglutinated by latex O157.

In the sewage with abundant original growth on the SMAC plates, the detection limit of PCR for stx_1 and stx_2 was 10^5 cfu/g, corresponding to 1000 cfu on the SMAC plate). By latex agglutination the O157 control strain was found on the SMAC plate corresponding to about 10^7 cfu/g.

The mTSB cultures of all spiked sewage samples were negative for stx_1 and stx_2 in PCR and also in the latex agglutination tests. On all SMAC plates, mold growth was seen.

The vast diversity of the sewage types in our study complicated the PCR detection of stx_1 and stx_2, as shown by the validation of this method. In fact, each individual sewage type should have been validated separately. However, of all sewage types two were chosen for the validation. These samples represented two extremes in their microbial contents: scanty (01/4010) growth and abundant (01/4109) growth on SMAC. The validation showed that the number of at least 1000 to 10,000 cfu/g of sewage was needed to detect STEC bacteria by PCR in these sewage samples. Occasionally STEC was also detected in a spiked sample containing only 10 cfu/g of sewage. However, we had used solid sewage samples (10 g) in the validation, which may have caused uneven distribution of the spiked STEC bacteria (100 µl) within the sample. In liquid samples, the

spread of bacteria would probably have been even. This suggests that the consistency of the sewage may have a dramatic effect on the detection of the stx_1 and stx_2 genes. Unfortunately, the same sludges were not used for the validations of IMS for STEC O157 (see validation of methods: Appendix 3.2) and PCR for all STEC.

3.2 Validation of the culturing methods for the detection of Listeria monocytogenes and Shiga-toxigenic Escherichia coli (STEC) O157 from sewage sludge and sludge products.

Tuula Johansson, National Veterinary and Food Research Institute, EELA, Department of Bacteriology/Food Microbiology

Listeria monocytogenes

Method

ISO 11290-1:1996, modified (more detailed description in the text, pages 17-18).

Procedure

Four sewage sludge samples, detected as being negative for *L. monocytogenes* in the study, were used as matrices for the validation. One of the samples was raw sludge (01/282), two were composts 30 weeks old (01/3959 and 01/3805) and the fourth was outfeed (01/515).

The deep-frozen (-20 °C) samples were thawed and weighed into portions of 25 g. The portions were inoculated with an overnight (24 h) TSB-culture of *L. monocytogenes* serotype 1/2 isolated from a sludge sample (EELA L1081). One ml of each of the appropriate dilutions of the TSB-culture containing *L. monocytogenes* 3.2 x 10^8 cfu/ml was added to the sample portions to obtain the levels of 3 cfu/25 g, 30 cfu/25 g and 300 cfu/25 g in duplicates (n=22). However, one of the compost samples and the sample of outfeed with the lowest inoculation level were analysed without replicates. In addition, an uninoculated sample was analysed from each sample matrix to show the absence of *L. monocytogenes* in the original samples (n= 4).

Results

L. monocytogenes was not detected in the uninoculated samples, excluding the sample of outfeed and compost. The level of *L. monocytogenes* in this sample was probably very low, because the sample was detected as being negative during the study.

The method detected as positive all the sample matrixes with the levels of 300 cfu (n=8) and 30 cfu (n=8) of *L. monocytogenes* in 25 g and the compost samples with the level of 3 cfu (n=3) of *L. monocytogenes* in 25 g. The method did not detect *L. monocytogenes* in the samples of raw sludge (n=2) and outfeed (n=1) inoculated with the level of 3 cfu/25 g.

Oh the basis of on the narrow β-haemolytic zone around the colonies with a typical appearance *L. monocytogenes* could be distinguished from the other *Listeria* species on improved LMBA, contrary to the PALCAM medium. Overgrowth by competing flora, especially by *Bacillus* species on both LMBA and PALCAM interfered with the detection.

Shiga-toxigenic *Escherichia coli* (STEC) O157

Method

ISO 16654:2001, modified (more detailed description in the text, page 19).

Procedure

Three sewage sludge samples, detected as being negative for STEC O157 in the study, were used as matrices for the validation. Two of the samples were raw sludge (01/1099, 01/1242) and the third was a composite sample of two outfeed samples (01/1500, 01/1295).

The deep-frozen (-20 °C) samples were thawed and weighed into portions of 25 g. The portions were inoculated with an overnight (24 h) TSB-culture of non-pathogenic STEC O157 (EELA381). One ml of each of

the appropriate dilutions of the TSB-culture containing 9.3 x 10^8 cfu/ml of STEC O157 were added to the sample portions to obtain the levels of 9 cfu/25 g, 90 cfu/25 g and 900 cfu/25 g in triplicates (n=27). In addition, an uninoculated sample was analysed from each sample matrix to show the absence of STEC O157 in the original samples (n=3).

Results

STEC O157 was not detected in the uninoculated samples.

The method detected as positive all the samples with the levels 900 cfu, 90 cfu and 9 cfu of STEC O157 in 25 g, after enrichment periods of both 6 h and 24 h. After 6 h enrichment STEC O157 grew as pure culture from almost all the samples, and the growth was heavy. When the enrichment period was lengthened from 6 h to 24 h, the number of STEC O157 colonies mainly decreased on both SMAC and CT-SMAC. After the enrichment period of 24 h more competing bacteria grew on the selective plates, especially on SMAC, compared with the enrichment period of 6 h, and STEC O157 was detected from composted sludge samples with all of the inoculation levels after 24 h on CT-SMAC only. Competing flora, especially *Pseudomas* spp. with the same colony morphology as *E. coli* O157 interfered with the detection on both SMAC and CT-SMAC.

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